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CORRECTIONS

Volume 37, 1959

Page 1318. In the headnote of Table II, (P) after "hypophysectomized" should read (H).

Volume 38, 1960

Page 505. In the paragraph beginning "As soon as they were dry, . . .", the second sentence should read: "They were then sprayed on the other side with diazotized sulphanilic acid (DSA) prepared, at 0° C, by mixing one volume of a solution of sulphanilic acid (0.9 g) in water (1 l.) containing concentrated hydrochloric acid (9 ml) with one volume of aqueous sodium nitrite (0.05%), and 15 minutes later, three volumes of aqueous potassium carbonate (4.0%)".

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VOLUME 38 JUNE 1960 NUMBER 6

THE EFFECT OF INTERMITTENT INDOOR COLD EXPOSURE ON WHITE RATS[1]

O. HÉROUX

With the technical assistance of DONNA WRIGHT

Abstract

Recently it has been shown that when white rats are kept in individual cages and exposed indoors to constant cold temperature or when they are exposed in groups to the outdoor winter conditions, they develop similar degrees of cold resistance and similar metabolic adjustments but they differ in endocrine, insulative, and peripheral adjustments.

In an attempt to determine whether variations in temperature alone could be responsible for the type of adjustments developed outdoors, white rats were kept at 30° C for 4 weeks but exposed to 6° C for a few hours every day. Through these intermittent cold exposures, the animals developed the same type of acclimation as the one produced indoors by continuous exposure to a constant temperature, but to a lesser degree. Adrenals tended to hypertrophy, body and muscle growth tended to decrease, and over-all insulation appeared to decrease. In contrast to continuously exposed rats, however, they showed no cold injury, no change in ear vascularization, and no thickening of the ear epidermis, probably because the skin was never cooled long enough at any given time for the cold temperature to produce cellular alterations that could not be corrected during the warm periods.

Introduction

Recent observations have revealed that in white rats climatic adjustments are not identical with those induced in the laboratory by continuous exposure to a constant temperature (1 – 4).

Under both the fluctuating environmental conditions prevailing outdoors during the winter and the conditions of constant cold temperature indoors, white rats increased their cold resistance in the same manner through a non-shivering mechanism of heat production that provided them with a much greater capacity for heat production than their controls.

This greater heat production capacity was obtained, however, through different endocrine, insulative, and peripheral adjustments under the outdoor than under the indoor conditions. The hyperplastic adrenals from the indoor cold-acclimated rats secreted steroids in vitro at a slower rate than the adrenals of their 30° C control rats, the thyroids appeared histologically more active, the total insulation was reduced, and cold acclimation was accompanied by

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increased ear vascularization, thickening of the ear epidermis, and extensive cold injury. In the outdoor winter rats, the adrenals were not hyperplastic but in vitro they secreted at a faster rate than the adrenals of the summer controls, and their thyroids showed histological signs of a greatly reduced state of activity. The total insulation was increased, there was no cold injury, no thickening of the ear epidermis, and no increase above the summer rats in the number of capillaries in the ear.

The constancy of the indoor cold temperature might be responsible for the cold injury and the reduced total insulation. However, other variable factors encountered outdoors, beside temperature, such as light and huddling of the grouped animals, might also influence the development of physiological and anatomical adjustments that lead to an increase in insulation and a reduction in cold injury.

In an attempt to determine whether variation in temperature alone could explain these different types of adjustments, white rats were kept at 30° C for 4 weeks, but exposed to 6° C for a few hours every day. Their degree and type of adaptation was estimated by their body growth, muscle and adrenal weight, ear vascularization, metabolic rate at different temperatures, peripheral cold injury, and finally their survival time at −26° C.

Experimental Procedures

Fifty Sprague–Dawley male rats (mean weight 214 g, range 171–251 g) were randomly assigned to three groups, 20 rats to group I kept at 30° C for 4 weeks, 19 rats to group II kept at 30° C except for intermittent exposure to cold (4 hours a day at 15° C during the first 3 days, 4 hours a day to 6° C for the following 3 days, and 6 hours a day to 6° C for 31 days), and finally 11 rats to group III kept at 6° C for 5 weeks. The rats were kept in individual cages and Master Fox Chow and tap water were supplied ad libitum. Artificial light was provided for the three groups from 8.00 a.m. to 8.00 p.m.

Gross body weight of each rat was recorded weekly. Oxygen consumption was measured, at 30° C, +6° C, −6° C, with an open circuit using the Pauling Oxygen analyzer (Beckman Model C). After all signs of cold injury on ears, tail, or penis were recorded, the animals were killed by an excess of ether. The adrenals and the right leg muscles (gastrocnemius, plantaris, soleus) were dissected and weighed on a torsion balance. The muscles were desiccated in an oven at 80° C and then transferred to a micro-Kjeldahl flask for nitrogen analysis; the water content was obtained by the difference between the wet and dry weight of the muscle, the lipid content was obtained by the difference between the dry weight and the protein content (nitrogen × 6.25), considering the carbohydrate and ash content as negligible. The left ear was dissected and put in a 10% formalin solution; after 24 hours' fixation, the tissues were sectioned, stained with benzidine for capillaries, and counted according to the methods described in a previous paper (5). One section of each ear was stained with hematoxylin–eosin and the thickness of the epidermis was measured with an eyepiece micrometer. Finally, on nine rats from each group, the degree of cold resistance was measured by their survival time at −26° C.

Results

In the three groups of rats, muscle weights, water, and protein contents of muscles and adrenal weights were correlated with body weight. Each gram increase in body weight was associated with a corresponding average increase of 5.8 ± 1.2 mg wet muscle, 4.32 ± 0.86 mg water, 1.36 ± 0.27 mg protein, and 0.202 ± 0.051 mg adrenal weight. In all cases, this relationship achieved the 1% level of statistical significance. These organ weights of the different groups were therefore compared on the basis of the same body weight (338 g).

In contrast to the rats exposed continually to cold, the ones exposed intermittently did not differ significantly from the controls in body and muscle weights (except for the lipid content of the muscles), in adrenal weight, thickness of ear epidermis, or number of capillaries in ears (Table I). Macroscopically, the ears, tail, and penis showed no signs of cold injury and the examination of the ear sections confirmed this gross observation since there was not even the slightest degree of oedema, one of the first signs of cold injury.

TABLE I

Effect of continuous and intermittent exposure of white rats to 6° C for 4 – 5 weeks on different parameters

Parameters	Acclimation groups		
	I 30° C	II 30° C – 6° C	III 6° C
Survival time, min	180 (9)†	880 (9)	3447 (9)
Final body weight, g	329 ± 6.9 (20)	312 ± 7.0 (19)	287 ± 9.2 (11)**
Leg muscle‡			
Total weight, mg	2062 ±54 (7)	1973 ±49 (8)	1748 ±67 (5)**
Water, mg	1554 ±41 (7)	1490 ±37 (8)	1307 ±51 (5)**
Protein, mg	437 ±13 (7)	428 ±12 (8)	402 ±16 (5)
Lipid, mg	72 ± 4.1 (7)	55.7 ± 3.8 (8)**	36.6 ± 4.8 (5)**
Adrenal weight, mg‡	34.6 ± 2.5 (7)	40.3 ± 2.3 (8)	54.8 ± 3.1 (5)**
Cold injuries	0	0	+++
Thickness of ear epidermis, μ	21.2 ± 0.8 (8)	22.5 ± 0.9 (8)	42.1 ± 4.2 (8)**
No. of capillaries/mm² in ears	18.9 ± 2.4 (8)	17.5 ± 2.4 (8)	60.6 ±11.2 (8)**

†No. of animals in parentheses.
‡Average weights at corresponding body weights (regressed weights).
**Significantly different from the control (30° C) (1% level).

In acclimated groups, log oxygen consumption was significantly though not closely ($r = 0.47$) correlated with log body weight (significant at 1% level), each unit increase in log body weight being associated with a corresponding average increase of 0.38 ± 0.15, 0.46 ± 0.20, and 0.64 ± 0.23 units log oxygen consumption at −6° C, +6° C, and +30° C exposure temperature respectively. Statistically, these regression coefficients did not differ significantly from one another, but they appeared to be linearly related to exposure temperature. To simplify the calculations, an average regression coefficient of 0.50 was used at all of the three test temperatures.

On this basis, the intermittently cold-exposed rats showed a significantly higher metabolic rate than the 30° C acclimated rats when the rate was measured at −6° C but not at +6° C or +30° C. The metabolic rate tended

to be higher, however, at any of the test temperatures (Fig. 1). Finally, the cold resistance of these intermittently cold-exposed rats was substantially increased since their survival time at −26° C was 880 minutes as compared to 180 minutes for the controls.

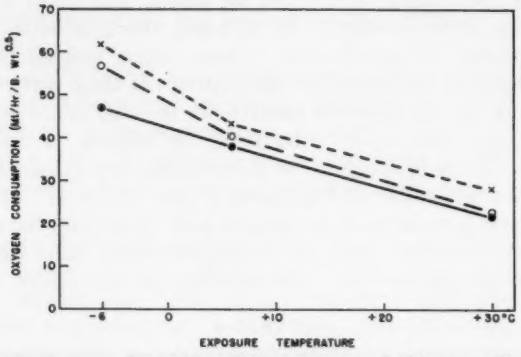

FIG. 1. Oxygen consumption of three different groups of white rats: ●——● rats kept at 30° C for 4 weeks, ○ - - - ○ rats kept at 30° C for 5 weeks but exposed to cold for a few hours every day, × - - - × rats kept at 6° C for 5 weeks.

Discussion

The preceding results definitely show that a certain degree of cold adaptation can be developed in rats that spend 18 out of 24 hours at 30° C every day and only 6 hours at 6° C. A similar observation has been reported recently by Molloy *et al.* (6).

However, in contrast to the rats constantly exposed to 6° C, but like the outdoor winter rats, the rats exposed intermittently to cold showed no sign of cold injury and no change in number of capillaries or thickness of the epidermis of the ear. These results confirm Meryman's conclusion that non-freezing cold injury occurs at temperatures below 10° C if the exposure period is long enough (7). Apparently under both conditions of exposure to 6° C for only 6 hours a day or of outdoor winter exposure with opportunities of the rats to huddle, the ear skin of the white rats was never cooled long enough at any given time for the cold temperature (−10° C on the average outdoors) to affect the cells permanently. As shown in a previous paper (2), the temperature coefficient of the skin is so high that under these intermittent conditions any cellular alteration due to cold was probably quickly corrected as soon as the skin was warmed, with the result that any enzymatic or metabolic disturbance never became severe enough to affect the cell to the point of no return. In the skin continuously exposed to 6° C, however, the cumulative cold damage could lead only to the death of the cell.

No statistically significant changes in body, muscle, and adrenal weights were observed in the rats intermittently exposed to cold, although all these organs changed in the same direction as they did in the rats continuously exposed to cold. These intermediate changes along with the intermediate

changes in metabolic rate and survival time suggest that the degree of metabolic adjustment was proportional to the degree of cold exposure. It is conceivable that similar changes would be obtained by continuous exposure at an intermediate cold temperature between 6° C and 30° C.

The intermittent exposure to cold therefore produced the same type of metabolic adaptation as found in rats kept constantly at 6° C for 4 weeks, although to a lesser degree. The way the environmental temperature was varied did not favor the development of the insulative type of adaptation (i.e., greater insulation and lower heat production in cold-acclimated animals) that seasonal climatic variations produce. Either the change in temperature has to be more frequent, more gradual, and the maximum and minimum temperatures have to be lowered gradually as in nature or else variable factors other than temperature or in conjunction with temperature cause the development of the greater insulation in the outdoor animals.

Observation of different species has shown the influence of light on fur (8, 9, 10) and recently Yeates (11) has shown that in cattle the switch from summer to winter coat was not associated with the environmental temperature but with length of day. It is therefore conceivable that variations in the conditions of light may be one factor responsible for the insulative type of adaptation observed in rats maintained outdoors. It must be noticed here that in the laboratory, periods of light exposure were the same for all groups.

Huddling is another factor that may have an important effect on the adjustments brought about by a cold environment. Separation of these different factors could be attempted: (*a*) by exposing grouped rats to a constant temperature such as −10° C indoors; (*b*) by exposing grouped rats in an outside shelter in which the fluctuating outdoor temperature would prevail but in which the intensity and duration of the illumination would be maintained at the same level as that received by the indoor rats; (*c*) by exposing grouped rats outdoors in a closed glass shelter in which the animals would be exposed to the normal fluctuating light conditions but maintained at a constant cold temperature; and finally (*d*) by exposing individual rats to outdoor conditions, although it is doubtful if the rats would survive the rigorous winter conditions under these circumstances.

References

1. O. HÉROUX, F. DEPOCAS, and J. S. HART. Can. J. Biochem. and Physiol. **37**, 473 (1959).
2. O. HÉROUX. Can. J. Biochem. and Physiol. **38**, 135 (1960).
3. O. HÉROUX and E. SCHÖNBAUM. Can. J. Biochem. and Physiol. **37**, 1255 (1959).
4. O. HÉROUX and J. CAMPBELL. Can. J. Biochem. and Physiol. **37**, 1263 (1959).
5. O. HÉROUX and J. ST. PIERRE. Am. J. Physiol. **188**, 163 (1957).
6. R. MOLLOY, D. NICHOLLS, W. FARRINGTON, and R. J. ROSSITER. Can. J. Biochem. and Physiol. **37**, 662 (1959).
7. H. T. MERYMAN. Research Report Project NM 000 018.01.07. Vol. 11. Naval Medical Research Institute. 1953. pp. 741–766.
8. J. BURKHARDT. J. Agr. Sci. **37**, 64 (1947).
9. J. HAMMOND, JR. J. Agr. Sci. **42**, 293 (1952).
10. D. S. HART. Nature (New Zealand), **171**, 133 (1953).
11. N. J. YEATES. J. Agr. Research, **6**, 891 (1955).

FORMATION IN VIVO OF 16-EPIESTRIOL AND 16-KETO-ESTRADIOL-17β FROM ESTRIOL BY THE LAYING HEN AND OCCURRENCE OF EQUOL IN HEN'S URINE AND FECES[1]

H. F. MacRae, D. G. Dale, and R. H. Common

Abstract

Estriol-16-C^{14} was administered intravenously to a laying hen furnished with exteriorized ureters. Urine and feces were collected separately and estrogens were extracted by a method that included acid hydrolysis but avoided a highly alkaline pH (> 10.5) for the extract. The urine secreted in the first 24-hour period after injection contained radioactive estriol, radioactive 16-ketoestradiol-17β, and radioactive 16-epiestriol. No other radioactive metabolite was detected in the urine. The feces excreted in the first 24-hour period after injection contained radioactive estriol and radioactive 16-epiestriol, but little, if any, radioactive 16-ketoestradiol-17β. The feces did contain small amounts of a radioactive metabolite less polar than estradiol-17β. Reasons are advanced in support of the view that this metabolite was present in consequence of bacterial action in gut or feces. Comparison of blackening of radioautograms showed that a high proportion of the injected estriol was excreted by the fecal route, and that the greatest single fraction of radioactivity present was in 16-epiestriol. The urine and feces for the second 24-hour period contained insignificant amounts of radioactive estrogens as compared with those excreted in the first 24-hour period.

The lipophilic and hydrophilic fractions of estrogen extracts both from urine and feces contained amounts of equol that were large relative to the amounts of the various estrogens excreted, as judged by staining of the chromatograms. Extracts from the first and from the second 24-hour periods contained about the same amount of equol.

Introduction

Studies on the metabolism of estrogens in vivo call for the determination of, first, the nature and, second, the amounts of the estrogens excreted. The following estrogens have been identified in human urine as normal excretory products: estriol (1); 16-epiestriol (2); 16α-hydroxyestrone (3); 16β-hydroxyestrone and 16-ketoestradiol-17β (4, 5); and 18-hydroxyestrone (6). The presence of 16-ketoestrone in human urine has been reported by Serchi (7) and by Slaunwhite and Sandberg (8). Kraychy and Gallagher (9) discovered that 2-methoxyestrone is a metabolite of estradiol-17β in the human, and Loke and Marrian (10) subsequently obtained 2-methoxyestrone from human pregnancy urine. Fishman and Gallagher (11) have detected 2-methoxyestriol as a metabolite of estradiol-17β in the human subject. Breuer and Pangels (12) have reported the isolation of 16,17-epiestriol (estriol-3,16β,17α) from human pregnancy urine. Breuer, Nocke, and Knuppen (13) have also reported the formation of 17-epiestriol from 16α-hydroxyestrone by human liver slices in vitro, but 17-epiestriol does not appear to have been isolated from urine as yet.

The occurrence of estrogens in species other than man has been reviewed by Dorfman (14). Here it may be noted that estrogens unsaturated in ring B, e.g., equilin and equilenin, appear to have been found almost exclusively in

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the horse. Estradiol-17α has been found in the urine of the pregnant mare (15), of the goat (16), and of the cow (17). Estradiol-17β was not detected in the urine of either goat or cow by Klyne and Wright (16, 17), although Gorsky and Erb (18) have reported the presence of both estradiol-17β and estradiol-17α in bovine placenta.

As regards avian species, Hurst, Kuksis, and Bendell (19) reported the presence of estradiol, estrone, and estriol in the droppings of the domestic fowl. Layne, Common, Maw, and Fraps (20) identified estradiol-17β, estrone, and estriol in the ovaries of laying hens. MacRae, Zaharia, and Common (21) isolated crystalline estradiol-17β from hens' droppings and characterized their isolate by infrared spectrophotometry. MacRae, Layne, and Common (22) injected radioactive estradiol-17β into a laying hen and subsequently detected radioactive estradiol, estrone, and estriol, as well as a fourth radioactive metabolite, in the gut and excrement. This fourth metabolite has been identified as 16-epiestriol (23).

The metabolic sequence of the estrogens has been reviewed by Marrian (24). The major sequence in the pregnant woman is believed to be estradiol $\rightarrow$ estrone $\rightarrow$ 16α-hydroxyestrone $\rightarrow$ estriol $\rightarrow$ 16-ketoestradiol-17β $\rightarrow$ 16-epiestriol. The demonstration of the presence of 16-epiestriol in human pregnancy urine by Marrian and Bauld (2) was of special interest because it vindicated the possibility of both 16α- and 16β-hydroxylations in estrogen metabolism.

Levitz, Spitzer, and Twombly (25) and Layne and Marrian (4) have demonstrated that the 16-ketoestradiol-17β found in pregnancy urine is not an artifact. The experiments of MacRae *et al.* (22) and of MacRae and Common (23) have shown that the laying hen can form estrone, estriol, and 16-epiestriol in vivo from estradiol-17β. In addition, Mitchell and Hobkirk (26) have shown that avian liver slices can convert estradiol-17β to estriol. MacRae *et al.* (23) have also noted that the ratio of the radioactive estriol to radioactive 16-epiestriol in the excreta of the laying hen following injection of radioactive estradiol-17β was much narrower than the ratio estriol:16-epiestriol reported for pregnancy urine (2). The narrower ratio in the excreta of the fowl accords with the view that much of the urinary estriol in the later stages of human pregnancy originates from the placenta (27).

In this connection, Ryan (28) has described the formation of estriol from neutral C-16-hydroxylated steroids by human placental tissue and has described the possibility that most of the estriol of human pregnancy urine may arise by some such metabolic route other than by C-16-hydroxylation of estradiol. If the metabolic pathway of estrogens in the laying hen is generally similar to that in the pregnant woman, then injection of radioactive estriol would be expected to give rise to radioactive 16-ketoestradiol-17β and radioactive 16-epiestriol in vivo, but to no other radioactive estrogen, provided that the extraction procedure had included acid hydrolysis which would destroy the unstable hydroxyestrones; and this argument, moreover, is independent of the possible origin of estrogens from non-aromatic steroids. The present paper describes an experiment designed to settle this point. It was considered

desirable to examine urine and feces separately because studies on the droppings are complicated by the possibility of secondary changes due to bacterial action.

Experimental Methods

The ureters of a Rhode Island Red laying hen were exteriorized by the operative procedure of Dixon and Wilkinson (29). The urine was collected by means of a harness and a latex rubber artificial vagina liner as described by Dixon (30), except that the liner was bent upwards and attached to the harness so as to form a collection sac. Urine and feces were collected several times daily and stored at −15° C. The bird remained in excellent condition. Laying was interrupted for 28 days after completion of the operation, but the hen laid well subsequently and was in lay at the time of the experiment. The long interval after operation obviated any possible effect that might have arisen in relation to postoperative diuresis. The diet was a commercial mixture for laying hens and contained dried alfalfa meal.

On the morning of the first day of experiment, 5.9 microcuries (0.27 mg) estriol-16-C^{14} in propylene glycol (1 ml) was injected into the wing vein. (The radioactive estriol was obtained from Dr. M. Levitz, Bellevue Medical Center, New York University, New York.) The urine and feces were collected separately for each of the next two succeeding 24-hour periods.

All four samples were worked up for estrogens by the following modification of Bauld's (31) procedure. The urine (or suspension of feces in water) was diluted to 500 ml with distilled water. To this was added 75 ml concentrated hydrochloric acid. The resultant solution was boiled under reflux for an hour, cooled, and extracted thrice with diethyl ether (peroxide-free) so that the final volume of the ethereal extract was approximately 500 ml. The ethereal extract was shaken with 100 ml concentrated carbonate buffer of pH 10.5. The aqueous phase was discarded and the ethereal extract was washed once with 15 ml *M* $NaHCO_3$ and subsequently with two successive 15-ml portions of distilled water. The ethereal layer was dried by anhydrous Na_2SO_4, and the ether removed under reduced pressure in a rotary evaporator. The residue was taken up in 2 ml of ethanol. In order to separate the estrogens into hydrophilic and lipophilic fractions, the solution was transferred to a separatory funnel with 25 ml benzene. Twenty-five milliliters of water was added to the mixture, which was then shaken gently so as to avoid emulsification. The benzene and aqueous phases were separated, and the benzene phase was extracted with two successive portions each of 25 ml water. The aqueous washings were added to the previously separated aqueous phase.

The benzene phase, containing the lipophilic estrogens, was evaporated under reduced pressure and taken up in methanol–dichloromethane for chromatography.

The combined aqueous phase and washings from the benzene phase was brought to pH 9.0 by very careful addition of NaOH solution (0.1 *N*), the pH being controlled by a pH meter. The resultant solution was extracted *at once* with diethyl ether. The ethereal solution of hydrophilic estrogens was dried over anhydrous Na_2SO_4. The ether was removed in a rotary evaporator and

the residue was taken up in methanol–dichloromethane for chromatography. Two chromatographic systems were used. System A was an absolute methanol – light petroleum (b.p. 80° – 100° C) system run at 34° C as described by Bush (32) and Layne *et al.* (20). System B was the chloroform–formamide system described by Layne and Marrian (5).

Experimental Results

Lipophilic Fraction from Urine

The lipophilic fraction from the urine sample for the first 24 hours was subjected to preliminary chromatography in system A. It yielded a single, broad, radioactive zone. The strip was cut in half longitudinally. The radioactive zone from the first half was eluted with methanol–dichloromethane. Carrier reference 16-ketoestradiol-17β (15 μg) was added to the eluate, which was then chromatographed in system B. Staining with diazotized sulphanilic acid (DSA) yielded two strongly stained zones (see Fig. 1, AS). Of these two

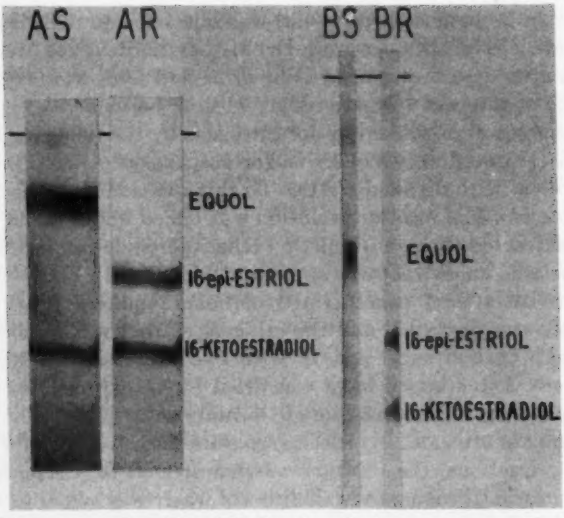

FIG. 1. Chromatography of lipophilic fraction of urinary estrogens of laying hen after injection with estriol-16-C^{14}.

AS. Chromatogram (chloroform–formamide) of first half of the lipophilic fraction to which had been added 15 μg reference 16-ketoestradiol-17β. Stained with DSA.

AR. Radioautogram of stained chromatogram AS. Note (*a*) congruence of staining and radioautogram of the 16-ketoestradiol-17β zone; (*b*) failure of radioactive 16-epiestriol zone to give perceptible staining with DSA; and (*c*) presence of heavily staining, non-radioactive equol zone.

BS. Part of chromatogram (chloroform–formamide) of lipophilic fraction. No addition of reference compounds. Stained with DSA.

BR. Radioautogram of stained chromatogram BS. Note that radioactive 16-ketoestradiol-17β zone did not show perceptible staining with DSA.

strongly stained zones, that with the greater R_f value was strongly radioactive and displayed congruence in shape and position of the staining with the blackening of the X-ray film (Fig. 1, AR). As will be shown below, the staining of this

zone was due entirely to the added reference ketoestradiol, so that this result provided an identification of the radioactive material as 16-ketoestradiol-17β. The strongly staining zone of lower R_f was devoid of radioactivity; it represented part of the urinary equol, a point which will be explained below. The radioautogram also revealed a strongly radioactive but non-staining zone with an R_f that corresponded with that of 16-epiestriol. This radioactive zone was eluted from an unstained portion of the chromatogram, and carrier reference 16-epiestriol was added to the eluate. The eluate was evaporated and the residue chromatographed in system B. Congruence of staining (DSA) and blackening of the radioautogram of the chromatogram confirmed the tentative identification as 16-epiestriol. Hence this zone represented the part of the radioactive 16-epiestriol which separated with the lipophilic fraction. Although 16-epiestriol, 16-ketoestradiol-17β, and equol had similar mobilities in system A, they could be separated cleanly in system B.

The radioactive zone of the second half of the chromatogram of the lipophilic fraction in system A was eluted and chromatographed in system B without the addition of reference estrogen. A strip 1 cm wide was cut longitudinally from the chromatogram, stained with DSA, and radioautographed. The staining yielded a strongly stained equol zone and four minor faintly staining zones (Fig. 1, BS). Comparison with the radioautogram (Fig. 1, BR) showed that none of these stained zones was radioactive, and that neither the radioactive 16-epiestriol nor the radioactive 16-ketoestradiol was present in sufficient amounts to give perceptible staining with DSA; and hence the observations shown in Fig. 1, AS and AR, constituted an identification of the 16-ketoestradiol-17β. The identity of the radioactive 16-epiestriol was confirmed by cutting out the appropriate zone from the remaining unstained portion of the chromatogram, eluting it with methanol–dichloromethane, adding reference 16-epiestriol (20 μg) and chromatographing the resultant solution in system B. Congruence of shape and position of the staining with DSA with blackening of the radioautogram confirmed the identity of the 16-epiestriol. These results showed that the injected radioactive estriol yielded, in this fraction, 16-epiestriol and 16-ketoestradiol-17β, but no other radioactive estrogen in amounts detectable by the methods used. (Any hydroxyestrones present would have been destroyed by the acid hydrolysis.)

Attention may now be given to the evidence for the identification of the equol zone. This zone stained strongly in circumstances where the amounts of the estrogens present were too minute to stain with DSA. This observation of itself suggested that the material was unlikely to be an estrogen. Furthermore, the mobility of the zone in system B did not correspond with that of any of the following: estradiol-17β, estrone, estriol, 16-epiestriol, or 2-methoxyestrone. Accordingly, the presumptive equol zone from the remaining unstained portion of the chromatogram of the lipophilic fraction was eluted with methanol–dichloromethane. The solution was evaporated and the residue was taken up in ethanol. The U.V. absorption spectrum was determined in a recording spectrophotometer (Warren Spectracord) and compared with the U.V. absorption spectrum of a sample of reference equol that had been isolated from goat's

urine. (The reference equol had been prepared by Dr. Wm. Klyne and was presented by Professor G. F. Marrian.) Both spectra displayed a smooth absorption band with a peak at 280 – 282 mμ and devoid of shoulders. The U.V. spectrometric evidence, therefore, was consistent with the identification of the material as equol. The alcoholic solution was evaporated to dryness, the residue was taken up in methanol–dichloromethane again, and portions of this solution were chromatographed in system B as follows:

Spot *a*, presumptive equol;

Spot *b*, presumptive equol plus reference equol (20 μg);

Spot *c*, reference equol (20 μg).

Staining with DSA showed that the presumptive equol and the reference equol had the same mobility in system A and that they gave the same hue with the stain. These observations left little doubt that the strongly staining, non-radioactive zone in the chromatogram of the lipophilic fraction was due to equol.

Equol was discovered by Marrian and Haslewood (33), who isolated it from mare's pregnancy urine. It has been found in the urine of the goat (16) and of the cow (17). Equol is thought to be a metabolite of plant isoflavones. Its occurrence in the urine and feces of the hen in the present work would be consistent with the fact that the diet contained dried alfalfa meal.

Hydrophilic Fraction from Urine

The hydrophilic fraction from the urine for the first 24 hours was subjected to preliminary chromatography in system A. It yielded one broad radioactive zone. This zone was eluted with methanol–dichloromethane and chromatographed again in system A. This time the single broad zone separated into

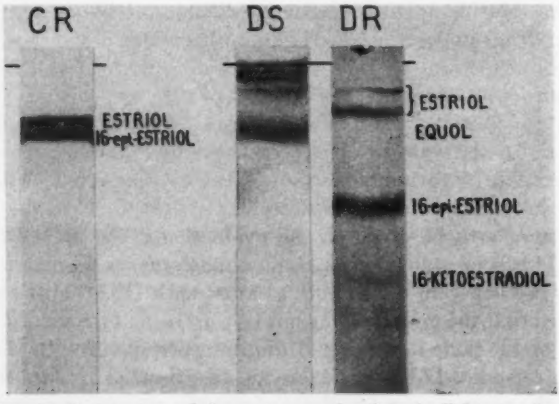

FIG. 2. CR. Radioautogram of chromatogram (methanol – light petroleum) of hydrophilic urinary estrogens of laying hen after injection of estriol-16-C^{14}.

DS. Chromatogram (chloroform–formamide) of radioactive material eluted from strip CR. Stained with DSA. Note strongly staining equol zone and other stained contaminants of lower R_f.

DR. Radioautogram of DS. Note (*a*) the appearance of two estriol zones due to interference of contaminants; (*b*) the absence of perceptible staining of the radioactive 16-epiestriol zone; and (*c*) the presence of traces of 16-ketoestradiol-17β.

two distinct radioactive zones (Fig. 2, CR). These zones corresponded in position with the expected positions of estriol and 16-epiestriol (23). The part of the strip that contained both radioactive zones was eluted and the eluate was chromatographed in system B. [The remaining parts of the strip were stained with DSA, which revealed strong staining at the edge immediately in advance of the 16-epiestriol zone. This staining was due to the trailing edge of the equol zone, for equol has a slightly lesser mobility than estriol in system A.]

The chromatogram of the radioactive zone in system B was stained with DSA and radioautographed, with the results shown in Fig. 2, DS and DR. There were five distinct stained zones. Of these, one was practically on the line of application and was not radioactive. Two others of low R_f value were also not radioactive. Presumably all three represented contaminating materials. A fourth, faintly staining zone carried some radioactivity. A fifth major, stained, non-radioactive zone was due to equol, which showed that this substance was present in relatively large amounts in the hydrophilic fraction as well as in the lipophilic fraction.

Four radioactive zones were present. The most strongly radioactive corresponded in R_f value with 16-epiestriol (Fig. 2, DR); and this observation confirmed the identification based on mobilities of the radioactive zones in system A (Fig. 2, CR). The next most strongly radioactive zone, which was not associated with staining, had an R_f corresponding with that of estriol and represented most of the radioactive estriol present. The third most radioactive zone corresponded with the fourth faintly staining zone. This relatively weak, narrow radioactive zone represented a fraction of the estriol which did not move with the main fraction of the estriol but with a contaminant of lower R_f than estriol. This effect, whereby estriol may give rise to two zones on chromatography of extracts of hens' excreta, has been observed previously (22). Chromatography of the hydrophilic fraction of an extract of feces to which some radioactive estriol had been added confirmed that such extracts may contain contaminants that cause estriol to give two zones on chromatography.

There was also a very faint radioactive zone in the position corresponding to 16-ketoestradiol-17β. This may have represented ketoestradiol that separated with the aqueous phase on partition, or it may have been due to entrainment of droplets of the benzene phase in the aqueous phase. In any event, the amount was extremely small.

Finally, attention is directed to the fact that estriol may be separated cleanly from equol in system B.

Lipophilic Fraction from Feces

The next stage was the examination of the feces for the first 24 hours. The lipophilic fraction was given a preliminary chromatography in system A and the chromatogram was radioautographed. It yielded one broad radioactive zone. This zone was eluted and the eluate was chromatographed in system B. A narrow strip was cut from the chromatogram, stained with DSA, and radioautographed. It yielded a faint stained zone in the position corresponding to equol ($R_f = 0.18$) and four other very faintly stained zones. None of these

five stained zones was radioactive. There was, however, (*a*) a strongly radioactive zone that corresponded in position to 16-epiestriol; (*b*) a very faintly radioactive zone corresponding in position to 16-ketoestradiol-17β; and (*c*) a faint, diffuse zone of radioactivity immediately behind the solvent front. This latter radioactive zone was not observed on chromatograms of either lipophilic or hydrophilic fractions from urine or of the hydrophilic fraction from feces, and its possible identity was, therefore, of considerable interest.

The presumptive 16-epiestriol zone (non-staining) was cut out from the main, unstained portion of the chromatogram, eluted, and reference 16-epiestriol was added to the eluate. The solution was chromatographed in system B. Congruence of staining and radioautogram of the resultant chromatogram confirmed the identification of the radioactive zone as 16-epiestriol.

The faint, diffuse, radioactive zone just behind the solvent front was eluted from the appropriate part of the main unstained portion of the chromatogram and reference estradiol-17β was added. The solution was then chromatographed in system A, stained with DSA, and radioautographed. A comparison of the staining and radioautogram showed that the major component of the radioactive material in question was not estradiol-17β but a less polar material, possibly estrone. It was not further identified.

Hydrophilic Fraction from Feces

The hydrophilic fraction from the feces yielded a single radioactive zone on preliminary chromatography in system A. This radioactive zone was eluted and the eluate was chromatographed in system B. The strip was stained with DSA and radioautographed. The radioautogram showed main and subsidiary estriol zones of low R_f, as had been observed for the hydrophilic fraction from the urine. There was also a strongly radioactive 16-epiestriol zone that did not stain, together with an extremely faint radioactive 16-ketoestradiol zone. A striking result was that the 16-epiestriol zone on the radioautogram was much blacker than the corresponding zone for the hydrophilic fraction of the urine. A strongly staining non-radioactive zone due to equol was also present.

Urinary and Fecal Samples for Second 24-hour Period

The samples of urine and feces for the second 24 hours yielded results which were in general similar to those described for the samples of the first 24-hour period, but the levels of radioactivity on the chromatograms were extremely low. The amounts of equol, however, were comparable with those found in the samples for the first 24 hours. By far the greater part of the radioactive urinary and fecal estrogens had obviously been excreted by the end of the first 24 hours.

Discussion

The results demonstrated that estriol is metabolized by the laying hen in vivo to 16-ketoestradiol-17β and 16-epiestriol. If the results for urine alone are considered, then it would appear that no other estrogen was formed in vivo from the injected estriol. This result is in agreement with the results and conclusions of Levitz *et al.* (34) regarding the metabolism of estriol in the human

subject. The results of the present experiment showed that the hen's feces contained considerable amounts of radioactive 16-epiestriol and little, if any, radioactive 16-ketoestradiol-17β. However, the feces also contained a detectable amount of a radioactive constituent less polar than estradiol-17β.

The latter observation does not necessarily invalidate the conclusion that the injected radioactive estriol gave rise in vivo to 16-epiestriol and 16-ketoestradiol-17β but to no other radioactive estrogen, apart from such as may have been destroyed by acid hydrolysis. Estrogens secreted via the alimentary tract are likely to be subject to bacterial action. It is possible and, indeed, likely that the appearance of an additional radioactive constituent in the feces in the present work was a consequence of the action of bacteria on the estriols or on 16-ketoestradiol-17β secreted in the bile or by the intestinal mucosa. The pregnant cow excretes more estrogen in the feces than in the urine (35, 36, 37), and most of the estrogenic activity of the cow's feces appears to be due to estradiol, although the major estrogen in the bile is estrone. Levin (35) originally suggested that this was a consequence of bacterial conversion of biliary estrone to estradiol. The situation in the fowl may be analogous, although the additional radioactive component in the hen's feces in this experiment was not estradiol-17β. On balance, the evidence favors the view that estriol gave rise to 16-epiestriol and 16-ketoestradiol-17β in vivo, but not to other estrogens, although one at least of these latter substances may have been formed from estriol or its metabolites in the intestinal canal.

The demonstration that equol is a possible major constituent of extracts of estrogens from either feces or urine points to the necessity of taking into account the possible presence of this substance in studies on the estrogens in avian excreta. It is fortunate that equol may be separated cleanly from estriol in the chloroform–formamide system.

Finally, the effects of contaminants on the chromatographic behavior of estriol is of technical importance in studies of the kind reported here.

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References

1. G. F. MARRIAN. Biochem. J. **24**, 435 (1930).
2. G. F. MARRIAN and W. S. BAULD. Biochem. J. **59**, 136 (1955).
3. G. F. MARRIAN, K. H. LOKE, E. J. D. WATSON, and M. PANATTONI. Biochem. J. **66**, 60 (1957).
4. D. S. LAYNE and G. F. MARRIAN. Nature, **182**, 50 (1958).
5. D. S. LAYNE and G. F. MARRIAN. Biochem. J. **70**, 244 (1958).
6. K. H. LOKE, G. F. MARRIAN, and E. J. D. WATSON. Biochem. J. **71**, 43 (1959).
7. G. SERCHI. Chimica (Milan), **8**, 9 (1953). (*Cited from* Chem. Abstr. **47**, 12496*c* (1953).)
8. W. R. SLAUNWHITE and A. A. SANDBERG. Arch. Biochem. Biophys. **63**, 478 (1956).
9. S. KRAYCHY and T. F. GALLAGHER. J. Am. Chem. Soc. **79**, 754 (1957).
10. K. H. LOKE and G. F. MARRIAN. Biochim. et Biophys. Acta, **27**, 213 (1958).
11. J. FISHMAN and T. F. GALLAGHER. Arch. Biochem. Biophys. **77**, 511 (1958).
12. H. BREUER and G. PANGELS. Biochim. et Biophys. Acta, **36**, 572 (1959).
13. H. BREUER, W. NOCKE, and R. KNUPPEN. Z. physiol. Chem. **311**, 275 (1958).
14. R. I. DORFMAN. Ann. Rev. Biochem. **26**, 523 (1957).
15. H. HIRSCHMANN and O. WINTERSTEINER. J. Biol. Chem. **122**, 303 (1937–1938).
16. W. KLYNE and A. A. WRIGHT. Biochem. J. **66**, 92 (1957).
17. W. KLYNE and A. A. WRIGHT. J. Endocrinol. **18**, 32 (1959).
18. J. GORSKY and R. E. ERB. Endocrinology, **64**, 707 (1959).
19. R. O. HURST, A. KUKSIS, and J. F. BENDELL. Can. J. Biochem. and Physiol. **35**, 637 (1957).
20. D. S. LAYNE, R. H. COMMON, W. A. MAW, and R. M. FRAPS. Nature, **181**, 351 (1958).
21. H. F. MACRAE, W. ZAHARIA, and R. H. COMMON. Poultry Sci. **38**, 318 (1959).
22. H. F. MACRAE, D. S. LAYNE, and R. H. COMMON. Poultry Sci. **38**, 684 (1959).
23. H. F. MACRAE and R. H. COMMON. Poultry Sci. In press (1960).
24. G. F. MARRIAN. Proc. Intern. Congr. Biochem. 4th Congr. Vienna. 1958.
25. M. LEVITZ, J. R. SPITZER, and G. H. TWOMBLY. J. Biol. Chem. **222**, 281 (1956).
26. S. L. MITCHELL and R. HOBKIRK. Biochem. and Biophys. Research Communication, **1**, 72 (1959).
27. R. C. MERRILL. Physiol. Revs. **38**, 463 (1958).
28. K. J. RYAN. J. Biol. Chem. **234**, 2006 (1959).
29. J. M. DIXON and W. S. WILKINSON. Am. J. Vet. Research, **18**, 665 (1957).
30. J. M. DIXON. Poultry Sci. **37**, 410 (1958).
31. W. S. BAULD and R. M. GREENWAY. Methods of biochemical analysis. Vol. 5. *Edited by* D. Glick. Interscience Publishers, Inc., New York. 1957.
32. I. E. BUSH. Biochem. J. **50**, 370 (1952).
33. G. F. MARRIAN and G. A. D. HASLEWOOD. Biochem. J. **26**, 1227 (1932).
34. M. LEVITZ, J. R. SPITZER, and G. H. TWOMBLY. J. Biol. Chem. **231**, 787 (1958).
35. L. LEVIN. J. Biol. Chem. **157**, 407 (1945).
36. W. H. PEARLMANN, A. E. RAKOFF, A. CANTAROW, and K. E. PASCHKIS. J. Biol. Chem. **170**, 173 (1947).
37. T. EL-ATTAR and C. W. TURNER. University of Missouri Agricultural Experimental Station. Bulletin No. 641. 1957.

STUDIES ON WHEAT PLANTS USING CARBON-14 COMPOUNDS

XII. UTILIZATION OF SERINE-C^{14} WITH SPECIAL REFERENCE TO GLYCINE LABELLING[1]

R. NATH[2] AND W. B. MCCONNELL

Abstract

Serine-C^{14} (uniformly labelled) was injected into the top internode of wheat plant stems and the distribution of carbon-14 in the mature plants (harvested 24 days after injection) was studied. Fifty-six per cent of the carbon-14 injected was found in upper plant parts but only trace amounts occurred below the top internode. The kernels contained 48% of the tracer injected while only 4% remained in the stem. Gluten had the highest specific activity of any major kernel component, serine and glycine accounting for one third of the total carbon-14 of the protein. The high specific activity of serine in gluten indicates its direct incorporation into kernel proteins. The specific activity of the glycine was almost equal to that of serine. Since similar experiments with glycine-1-C^{14} have yielded gluten with highly radioactive serine-1-C^{14}, the ready interconvertibility of these two amino acids is demonstrated. The results indicate that interconversion takes place, at least in part, by the reversible condensation of "active" formate with carbon-2 of the glycine but that alternate pathways may also operate.

Introduction

The interconversion of glycine and serine in biological systems has been extensively studied in recent years (1). The occurrence of this interconversion in animals and in microorganisms has been well established and a good deal is known of intermediates formed and of enzymes involved in the transformations (2). The quantitative aspects of the interconversion have also been investigated, especially in animals (3). Relatively little is known, however, about the interrelation of these amino acids in higher plants (4, 5).

An earlier publication in this series described the incorporation of glycine-1-C^{14} and formate-C^{14} into the tissues of maturing wheat plants (6). Serine isolated from gluten following injection of either of these substances was of comparatively high specific activity. Furthermore, the localization of carbon-14 in the skeleton of this serine was such as to suggest that the latter had been formed from the glycine moiety by condensation with the carbon of formate. The present study shows that serine-UL-C^{14}, when injected into the stems of maturing wheat plants, is readily converted to glycine. Other aspects of the serine-C^{14} utilization are also discussed.

Experimental Methods

Wheat plants used in the present experiments were seeded in the greenhouse on March 8, injected with tracer July 4, and harvested fully mature on July 28. The plants were grown in subirrigated gravel culture using a Hoagland's nutrient solution and without the use of artificial light. Day temperatures were

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[2]National Research Council of Canada Postdoctorate Fellow 1957–1959.

68° F; night temperatures 58° F. Plant growth was vigorous, the height and yield being markedly in excess of those used in similar experiments with plants grown in the field (6, 7). Maturation was slower than under natural conditions, the period from seeding to harvest being 142 days compared with periods of about 100 days for maturation outdoors (6). On July 4 (24 days before harvesting) 5 μc (0.11 mg) of uniformly labelled serine-C^{14} in 0.1 ml of aqueous solution was injected into the top internode of single tillers on each of 10 different wheat plants using methods previously described (7, 8). The plants were very well developed at the time, and showed signs of maturing. Kernels were fully developed in size and described as being in the dough stage.

The kernels were separated from the tillers collected, pooled, and ground to pass a 40-mesh screen. A similar procedure was used to obtain ground samples of chaff, rachis, and stem portion above the top node. Because of the protected growth conditions many anthers still adhered to the mature heads and a sample of these was also collected and ground. Methods of preparing the samples (7), fractionating the wheat meal into major components (7), and isolating and degrading the amino acids of the proteins were as previously used in this laboratory (8, 9, 10). Carbon-14 was determined by counting carbon dioxide obtained by wet combustion of labelled materials with a vibrating reed electrometer (Dynacon Nuclear-Chicago). The methyl group of methionine was isolated as tetramethyl ammonium iodate by the method previously used (6). A workable quantity of methionine was obtained from another hydrolyzate of the protein by the addition of 5 mg of inactive methionine prior to its chromatographic isolation. This methionine was purified by chromatography on Whatman No. 3 filter paper, before being used for degradation experiments.

Results and Discussion

Table I gives the carbon-14 content of the plant parts examined. About 56% of the carbon-14 introduced was recovered in the parts harvested, seven-eighths of this having been deposited in the kernels. These observations are

TABLE I

The carbon-14 content of plant parts examined

Plant part	Weight, g/plant	Specific activity, mμc/mmole CO_2	Carbon-14 content	
			μc	% C^{14} injected*
Kernels	2.174	31.3	2.42	48.50
Chaff	.374	7.67	.106	2.12
Rachis	.084	12.46	.041	.81
Stem	.517	11.80	.216	4.31
Anthers	.009	2.47	.001	.02
			2.782	55.76

*Serine-C^{14} was injected into the plants at the rate of 5 μc per plant.

similar to those obtained with other tracers using plants grown in the fields (6, 8, 9). It should be stated that the yields of plant material were much higher than for plants produced outdoors. The weight of kernels recovered, for

example, was 2.17 g per head compared with from 0.80 to 1.20 g per head in earlier experiments. Many anthers adhered to the heads of indoor-grown plants, and a sample of these was collected and analyzed for carbon-14. Since the plants were well developed and kernels fully formed at the time tracer was introduced into the stem, it is noteworthy that the anthers acquired a measurable radioactivity. They were not further analyzed.

Table II shows the distribution of carbon-14 in kernel components. The yields of the fractions per gram of kernels are in good agreement with those obtained in previous experiments. The pattern of carbon-14 incorporation into

TABLE II

Distribution of carbon-14 in kernel components

Kernel component	Yield from 1 g	Specific activity, mμc/mmole CO_2	μc	Carbon-14, % of kernel C^{14}
Kernels	1.00	31.3	1.11	100
Starch	.580	26.8	.576	51.8
Gluten	.130	54.2	.316	28.5
Salt-soluble protein	.034	34.0	.009	.8
Lipid	.021	29.2	.041	3.7
Bran	.111	22.5	.096	8.7
	.876		1.038	93.5

the components is also similar to that obtained with glycine-1-C^{14}. The proteins as would be expected had the highest specific activity of the fractions isolated. However, starch had an appreciable specific activity and, since it is the major component of the kernels, contains about half of the kernel carbon-14. Serine is thus very glycogenic in the wheat plant. Kernel weight and carbon-14 not accounted for are presumed to have been discarded with water-soluble "waste" material.

The labelling of a number of amino acids from the gluten is given in Table III. The two most radioactive amino acids isolated were serine and glycine, the latter being only about 15% less active than serine. Methionine and histidine were next in order of specific activity. The specific activity of other amino acids was comparatively low and of about the same order of magnitude as that of starch and other materials. The specific activities of carbons 1 and 5 of the major amino acid, glutamic acid, were 31 and 20 mμc/mmole CO_2 respectively. The average specific activity of all carbons is 39 mμc/mmole CO_2 and hence it is shown that the tracer has been concentrated to some extent in internal carbons.

The serine-C^{14} isolated from the hydrolyzate accounted for about 21% of carbon-14 in the gluten. This corresponds to 6.4% of the carbon-14 of the kernels and 3.1% of the carbon-14 administered to the stems. Similarly glycine contained about 12% of the carbon-14 of the protein, equivalent to 3.7% of kernel carbon-14 and 1.8% of carbon-14 introduced. Serine and glycine thus account for about 33% of carbon-14 in the protein while they comprise only 8% of the total weight.

The present results, coupled with the previous observation that carbon-1 of serine is extensively labelled following injection of glycine-1-C^{14}, demonstrate

TABLE III

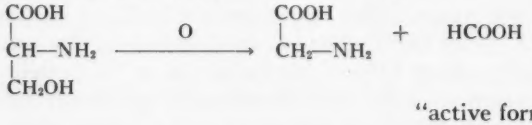
Carbon-14 content of amino acids of wheat gluten following administration of serine-UL-C^{14}

Amino acid	Specific activity, mμc/mmole CO_2	
	Total	Ninhydrin-C
Glutamic acid	39	31
Arginine	27	20
Proline	48	32
Aspartic acid	26	32
Threonine	29	26
Alanine	44	28
Serine	446	304
Glycine	386	293
Leucine	60	43
Isoleucine	28	16
Valine	31	19
Histidine	85	36
Lysine	18	26
Tyrosine	45	20
Phenylalanine	53	32
Methionine	157	70

that there is ready interconversion of these two amino acids in the maturing wheat plant. It would appear, in fact, that the labelling of serine and glycine of the wheat gluten is not markedly dependent upon whether tracer is administered as glycine-C^{14} or as serine-C^{14}. Thus when glycine-1-C^{14} was given (6), about 2.8% and 3.3% of the carbon-14 appeared in gluten as serine and as glycine respectively. It is found from the present data (6, 11) that 3.1% of the carbon-14 injected as serine-UL-C^{14} was recovered in serine of the gluten, while approximately 2.8% of the serine injected was converted to glycine. The latter value is calculated on the assumption that two of the three serine carbons appear in glycine.

The distribution of carbon-14 in the serine and glycine isolated from the wheat gluten (Fig. 1) shows preferential localization of carbon-14 in carbon-2 of both amino acids. It may also be noted that carbon-3 of the serine is more highly radioactive than is carbon-1.

It has been shown, in work with other systems, that the methyl group of methionine (1, 12) and the amidine group of histidine (13, 14) can be derived from "active formate" and hence from carbon-3 of serine by participation of the following general reaction (12).



"active formate"

Incorporation of appreciable serine carbon-14 into methionine and histidine in the present experiment thus indicates that the above reaction plays an important role in the conversion of serine to glycine in wheat. It also follows

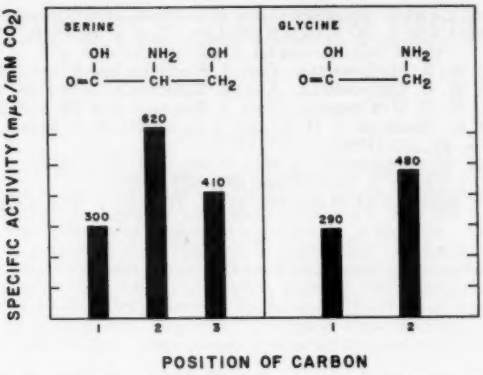


FIG. 1. Distribution of carbon-14 in serine and glycine from gluten.

from the above discussion that methionine carbon-14 should be localized in the methyl group. Although the amount of labelled methionine available was too small for precise degradative studies it was estimated that the specific activity of its methyl group was between 400 and 600 mμc/mmole CO_2. The approximation suffices to show preferential labelling of this group and indicates in fact that it has a specific activity comparable to carbon-3 of serine re-isolated from the gluten. It is probable that the amidine carbon of histidine is also preferentially labelled but because of insufficient material this point was not examined.

The above discussion does not rule out the possibility that serine is also converted to glycine in maturing wheat by other reactions. On the contrary, the high specific activity found in carbon-2 of gluten serine makes it probable that there is some cycling in which serine is decarboxylated and converted to glycine by way of intermediates such as ethanolamine. It is even possible that interconversion occurs in part by transfer of carbon-3 of serine to glycine without net synthesis or loss of glycine or serine (2).

Clearly, additional work is required to satisfactorily establish the mechanism of serine–glycine interconversion in wheat and, if more than one metabolic pathway is involved, to determine their relative importance.

Acknowledgments

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References

1. A. MEISTER. Biochemistry of the amino acids. Academic Press, Inc., New York. 1957. p. 271.
2. W. SAKAMI. *In* Symposium on amino acid metabolism. *Edited by* W. D. McElroy and H. B. Glass. John Hopkins Press, Baltimore, Maryland. 1955. p. 658.
3. K. WHITE. Arch. Biochem. Biophys. **75**, 215 (1958).
4. A. H. W. HAUSCHILD. Can. J. Biochem. and Physiol. **37**, 887 (1959).
5. A. P. WILKINSON and P. D. DAVIES. Nature, **181**, 1070 (1958).

6. W. B. McConnell and E. Bilinski. Can. J. Biochem. and Physiol. **37**, 549 (1959).
7. W. B. McConnell and L. K. Ramachandran. Can. J. Biochem. and Physiol. **34**, 180 (1956).
8. E. Bilinski and W. B. McConnell. Can. J. Biochem. and Physiol. **35**, 357 (1957).
9. E. Bilinski and W. B. McConnell. Can. J. Biochem. and Physiol. **36**, 881 (1958).
10. E. Bilinski and W. B. McConnell. Can. J. Biochem. and Physiol. **35**, 365 (1957).
11. J. W. Pence, D. K. Mecham, A. H. Elder, J. C. Lewis, N. S. Snell, and H. S. Olcott. Cereal Chem. **27**, 335 (1950).
12. S. Nakao and D. W. Greenberg. J. Biol. Chem. **230**, 603 (1958).
13. L. Levy and M. J. Coon. J. Biol. Chem. **192**, 807 (1951).
14. H. Tabor, A. H. Mehler, O. Hayaishi, and J. White. J. Biol. Chem. **196**, 121 (1952).

OXIDATION OF SULPHYDRYL GROUPS IN DENATURED EGG ALBUMIN[1]

C. C. TSEN[2] AND A. L. TAPPEL

Abstract

The oxidation of dry denatured egg albumin and dry myosin by molecular oxygen indicates that the direct oxidation of dry proteins containing free sulphydryl groups is a general reaction. Cupric ion was the most effective catalyst for the oxidation of denatured egg albumin in solution. The rate of the oxidation was measured as a function of cupric ion concentration, pH, and temperature. Since the catalytic oxidations of denatured egg albumin, glutathione, and cysteine have very similar activation energies, the mechanism of copper-catalyzed sulphydryl oxidation may be the same for the three compounds. Under the conditions studied, only sulphydryl groups of denatured egg albumin were oxidized. The oxidation of these groups proceeded beyond the disulphide stage.

Introduction

The sulphydryl groups of proteins undergo direct oxidation by molecular oxygen, both when the protein is dry and when in solution. This mode of oxidation in hemoglobin was studied previously (1). Denatured egg albumin was chosen as a protein for further study because it was found to be oxidized readily in aqueous solutions and this oxidation was accelerated by cupric ions. The results of the study are the subject of this communication.

Metal catalysis in the oxidation of sulphydryl groups of proteins was first observed by Hellerman *et al.* (2) when urease lost its activity by aeration in the presence of cupric ions. Weill and Caldwell (3) found that ferricyanide alone would not oxidize the sulphydryl group of amylase, but cupric ions catalyzed the oxidation. Robert (4–6) found that the oxidation of sulphydryl groups of alkali-denatured serum albumin was catalyzed by copper.

Experimental

Crystalline egg albumin (twice crystallized, salt-free) and L-histidine were purchased from Nutritional Biochemicals Corporation, L-tryptophan was obtained from Eastman Kodak Co., and cysteine hydrochloride (C.P.) from Fisher Scientific Co. All chemicals used were reagent grade. Water was redistilled in a glass apparatus. Myosin was prepared by the method of Szent-Györgyi (7). The dry denatured egg albumin was prepared by treating egg albumin with 6 *M* urea under a nitrogen atmosphere and then by lyophilizing. Both freeze-dried denatured egg albumin and myosin were equilibrated, before the oxidation, to 11 mm Hg water vapor pressure in a nitrogen atmosphere.

Oxygen absorption was measured manometrically. Unless otherwise stated, the reaction system in each Warburg flask consisted of 3.4×10^{-6} *M* egg albumin in 0.03 *M* tris-(hydroxymethyl)aminomethane buffer at pH 8.0 and 1×10^{-5} *M*

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cupric chloride. The flasks were wrapped with aluminum foil to exclude light, thus obviating any possible photoreactions. They were incubated at 37° C under an atmosphere of pure oxygen, while shaken at 60 oscillations per minute in the Warburg respirometer. The urea solution was introduced at zero time from a side arm of the flask to denature the egg albumin, liberate sulphydryl groups, and initiate oxidation simultaneously. The final concentration of urea was 6 *M* in the reaction system. If the concentration of urea higher than 6 *M* was used, the protein coagulated after the oxidation under our experimental condition.

Sulphydryl groups of the proteins were measured by the method of Benesch, Lardy, and Benesch (12) with the following modifications. The titration was carried out under an atmosphere of nitrogen to diminish the oxidation by air. A magnetic stirring apparatus was used to facilitate the mixing of the titration solution and the sample solution. This reduced the time required for the titration. The amperometric titration worked well for proteins in solution. However, after the long period of oxidation, the dry proteins were not completely dissolved in the titration mixture. In such cases, the protein was homogenized with the titration mixture under a nitrogen atmosphere. The homogenized solution was used for the amperometric titration. For inhibition studies, the albumin solution was first treated with the sulphydryl reagent and then denatured by urea in the Warburg flask under the same conditions of egg albumin oxidation.

Results and Discussion

Oxidation of Denatured Egg Albumin and Its Catalysis by Metal Ions

The results in Table I show that dry denatured egg albumin is directly oxidized by molecular oxygen. For comparison, another protein with available sulphydryl groups, myosin, was allowed to oxidize under identical conditions.

TABLE I

Oxidation of freeze-dried denatured egg albumin and myosin

Protein	Cupric chloride, % dry weight	Initial rate, ml O_2/g/hr	After 129 hr, ml O_2/g
Denatured egg albumin	0	0.024	0.82
Denatured egg albumin	1.1	0.032	1.14
Myosin	0	0.012	0.68
Myosin	1.1	0.031	1.34

$CuCl_2$ was added prior to freeze-drying. The freeze-dried proteins were oxidized at 37° C in oxygen.

Oxidation of these dry proteins was catalyzed when bound copper was present. Amperometric titration of these proteins after 129 hours' oxidation showed that all sulphydryl groups were oxidized. The oxidation of dry hemoglobin under these conditions is known to be due to sulphydryl oxidation which proceeds at an initial rate of 0.086 ml O_2/g/hour at 55° C (1). From these three examples it appears that the direct oxidation of dry proteins containing free sulphydryl groups is a general reaction. Aqueous denatured egg albumin was readily

oxidized at a constant initial rate of 0.09 mole oxygen/mole egg albumin/hour. Amperometric titration showed a decrease from 3.56 moles sulphydryl/mole denatured egg albumin before oxidation to 1.78 moles sulphydryl/mole denatured egg albumin after 4 hours of oxidation. Metal salts which are good catalysts for the oxidation of cysteine and glutathione were evaluated as catalysts for the oxidation of denatured egg albumin. The salts, Na_2SeO_3, $FeCl_3$, $MnSO_4$, and $CoCl_2$, at 1×10^{-5} M were not very effective catalysts as shown by their initial rates of oxygen absorption of 0.07, 0.10, 0.15, and 0.23 mole oxygen/mole egg albumin/hour, respectively. By comparison, $CuCl_2$ was a good catalyst with a rate of 0.81 mole oxygen/mole egg albumin/hour. In 4 hours of copper-catalyzed oxidation, the sulphydryl groups were almost completely oxidized; amperometric titration showed at the end of this period only 0.155 mole sulphydryl/mole denatured egg albumin.

The results of catalytic oxidation of denatured egg albumin over a wide range of $CuCl_2$ concentrations at pH 8.45 show in Table II that the rate increased with increasing $CuCl_2$ concentrations up to 1×10^{-5} M. Above this

TABLE II

Effect of copper concentration on the oxidation of denatured egg albumin

Concentration of $CuCl_2$, $M \times 10^6$	Initial rate, mole O_2/mole egg albumin/hr
0	0.19
1	0.29
5	0.76
10	1.19
100	0.69

concentration of $CuCl_2$, the denatured egg albumin coagulated and the oxidation rate was decreased. Thus, for a maximum oxidation rate a ratio of about 2.9 moles Cu:1 mole denatured egg albumin was required in our experiment. This was much lower than that of 1000 moles Cu:1 mole alkaline-denatured serum albumin found by Robert *et al.* (4–6).

Although copper may be bound to a number of functional groups in a protein, at low concentrations of copper the reaction with sulphydryl groups is most significant. At a high pH, co-ordination complexes between copper and nitrogen can also be formed (8). Since Robert *et al.* employed a high pH, much of the copper could have reacted with peptide bonds to form biuret-like complexes, and thus some of the Cu ions would have been bound and catalytic activity decreased. In our system, in addition to the denatured egg albumin, the urea and tris-(hydroxymethyl)aminomethane could react with copper. Therefore, the concentration of copper actually required for a maximum rate of oxidation might be less than 2.9 mole copper/mole denatured egg albumin. Kolthoff and Willeford (9) noted that the sulphydryl groups in native bovine serum albumin were oxidized by oxygen when the copper concentration was 1 mole copper/mole bovine serum albumin or greater.

Effect of pH and Temperature

The data in Table III show the effect of pH on the rate of oxidation of denatured egg albumin. At pH 5.90 or lower the egg albumin coagulated and there was no oxidation. The maximum rate obtained at pH 9.25 may be

TABLE III

Effect of pH on the oxidation of denatured egg albumin

pH (0.03 *M* Tris buffer)	Initial rate, mole O_2/mole egg albumin/hr
5.90	0
7.30	0.52
8.48	1.29
9.25	1.78
12.51	1.10

attributed to the fact that the sulphydryl group is most oxygen-reactive at these pH values near its pK_a of 10.3. At pH 12.51, the initial rate of oxidation was slow; however, its rate remained steady without showing any decline during a 3-hour oxidation period. This effect is probably due to the oxidation of other reactive amino acids such as tyrosine, tryptophan, and histidine of the protein, when the protein is in the alkaline solution.

The effect of temperature was determined at 17, 27, and 37° C and the initial rates for the oxidation of denatured egg albumin catalyzed by 1×10^{-5} *M* $CuCl_2$ were 6.77×10^{-3}, 1.37×10^{-2}, 2.60×10^{-2} mole oxygen/mole protein/minute, respectively. On the basis of the rates, an activation energy of 12.2 kcal/mole was obtained from the Arrhenius relationship. For comparison, 4.9×10^{-5} *M* cysteine in 0.1 *M* phosphate buffer, pH 7.2, was catalytically oxidized by 0.01 mole $CuCl_2$/mole cysteine and gave rates of 1.69×10^{-3}, 3.86×10^{-3}, and 7.49×10^{-3} mole oxygen/mole cysteine/minute at temperatures of 7, 17, and 27° C, respectively. These values likewise conformed to the Arrhenius relationship and gave an activation energy of 13.2 kcal/mole. For copper-catalyzed glutathione oxidation under similar conditions, the activation energy was previously found to be 13.2 kcal/mole (10). Thus we have three examples of sulphydryl oxidation catalyzed by copper which have very similar activation energies regardless of their differences in molecular size. This suggests that the mechanism of copper-catalyzed sulphydryl oxidation may be the same in denatured egg albumin, glutathione, and cysteine.

Oxidation of Sulphydryl and Other Groups in Egg Albumin

The results presented thus far show that the oxidation of these liberated sulphydryl groups is the main reaction. This oxidation of sulphydryl groups was determined by simultaneous measurements of oxygen absorption and amperometric titrations. The results in Fig. 1 show that for the first 50 minutes the oxygen absorbed is equal to the sulphydryl groups lost, assuming oxidation to a disulphide. After 50 minutes the amount of oxygen consumed is greater indicating that the sulphydryl groups are oxidized beyond the disulphide and (or) that other groups in the protein are also oxidized. These two possibilities were evaluated by specifically blocking the sulphydryl groups of

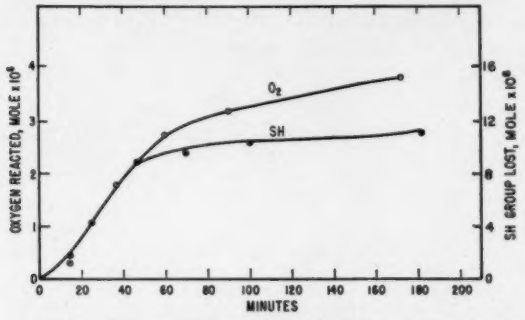

FIG. 1. The oxidation of denatured egg albumin. The amount of oxygen reacted was measured manometrically and the decrease in the number of sulphydryl groups was measured amperometrically. The pH was 7.80 and the other conditions are described in the experimental section.

denatured egg albumin with *p*-chloromercuribenzoate and mercuric chloride. *p*-Chloromercuribenzoate at 1, 2, and 4 moles/mole egg albumin gave increasing inhibition of the rate of oxidation. As a typical example, in 2 hours inhibition of the oxidation rate of denatured egg albumin was 38, 52, and 84% for 1, 2, and 4 moles *p*-chloromercuribenzoate/mole egg albumin, respectively. With 2 moles of mercuric chloride/mole egg albumin the oxidation was completely inhibited. The different degrees of inhibition showed that the sulphydryl groups of denatured egg albumin are chiefly responsible for its oxidation and that cysteine is the first amino acid in the protein to react with oxygen. This reactivity of cysteine was further evaluated by exposing the most reactive amino acids to oxygen under the conditions of egg albumin oxidation. The results in Table IV

TABLE IV

Oxidation of amino acids

Amino acid	Concentration, $M \times 10^5$	Oxidation at: 5 hours, mole O_2/mole amino acid	Oxidation at: 50 hours, mole O_2/mole amino acid
Cysteine	4.9	0.44	0.98
Tryptophan	15.7	0.025	0.053
Histidine	72.0	0.001	0.023

NOTE: The reaction system contained the amino acid, 6 *M* urea, 0.03 *M* tris-(hydroxymethyl)aminomethane buffer, pH 8.3–8.6, and $CuCl_2$ catalyst at 1 mole $CuCl_2$/100 moles amino acid. These amino acid solutions were oxidized at 37° C in oxygen.

show that histidine and tryptophan oxidize only slightly under these conditions. Cysteine oxidizes readily and is oxidized beyond cystine, probably to cysteine sulphinic acid, as the mole ratio of oxygen consumed/cysteine indicates. This result is in agreement with the recent finding of Povoledo *et al.* (13) that cupric ion catalyzes the oxidation of sulphydryl and disulphide groups and that cysteine sulphinic acid is the major oxidation product of cystine.

From the quantity of oxygen reacted with denatured egg albumin, the results of mercuric inhibitors, and the reactivity of cysteine with oxygen under these

conditions, it may be concluded that the sulphydryl groups of denatured egg albumin are oxidized beyond the disulphide.

References

1. C. C. TSEN and A. L. TAPPEL. Arch. Biochem. Biophys. **75**, 243 (1958).
2. L. HELLERMAN, M. E. PERKINS, and W. M. CLARK. Proc. Natl. Acad. Sci. **19**, 855 (1933).
3. C. E. WEILL and M. L. CALDWELL. J. Am. Chem. Soc. **67**, 214 (1945).
4. B. ROBERT. Bull. Soc. Chim. Biol. **36**, 253 (1954).
5. M. MACHEBOEUF and B. ROBERT. Bull. Soc. Chim. Biol. **35**, 399 (1953).
6. B. ROBERT, E. BARBU, and M. MACHEBOEUF. Bull. Soc. Chim. Biol. **36**, 271 (1954).
7. A. SZENT-GYÖRGYI. Chemistry of muscular contraction. 2nd ed. Academic Press, New York. 1951. p. 146.
8. I. M. KLOTZ and H. A. FIESS. J. Phys. & Colloid Chem. **55**, 101 (1951).
9. I. M. KOLTHOFF and B. R. WILLEFORD, JR. J. Am. Chem. Soc. **80**, 5673 (1958).
10. C. C. TSEN and A. L. TAPPEL. J. Biol. Chem. **233**, 1230 (1958).
11. F. W. PUTNAM. *In* The proteins. Vol. 1, Part B. *Edited by* H. Neurath and K. Bailey. Academic Press, New York. 1953. p. 883.
12. R. E. BENESCH, H. A. LARDY, and R. BENESCH. J. Biol. Chem. **216**, 663 (1955).
13. D. POVOLEDO, C. DE MARCO, and D. CAVALLINI. Italian J. Biochem. **7**, 78 (1958).

THE ACTION OF NORMAL AND ATYPICAL CHOLINESTERASE OF HUMAN SERUM UPON A SERIES OF ESTERS OF CHOLINE[1]

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Abstract

The results of a comparison of the actions of individual human sera support evidence previously obtained for the existence of two different types of pseudocholinesterase in man. Maximum rates of hydrolysis of choline esters by human sera containing the normal type of cholinesterase were two to four times greater than the maximum rates obtained with sera containing atypical esterase. Human sera with the normal type of cholinesterase hydrolyzed butyrylcholine at a considerably faster rate than pentanoylcholine; atypical esterase hydrolyzed pentanoylcholine at approximately the same rate as butyrylcholine. All Michaelis constants for the normal enzyme were lower than those for the atypical enzyme by a factor which varied from 1.4 to 6.4. The data were compatible with the assumption of a linear relationship between the log of the Michaelis constants and the number of acyl carbons in the choline esters, but the slopes differed significantly for the two enzymes. For a homologous series of choline esters, there was no correlation between rates of hydrolysis and Michaelis constants.

Evidence for the existence of an inherited atypical form of pseudocholinesterase in the serum of some individuals has been obtained by genetic (1) and statistical studies (2), and by investigations using inhibitors (3) and a series of different buffers (4). A differential electrophoretic mobility of the two enzymes can be achieved by adding inhibitors to the supporting medium (4) but the structural difference between the two types of enzyme is probably small.

About 3.8% of a population of European stock possess a mixture of the normal and the atypical esterase, while the exclusive occurrence of atypical esterase is rare. The best estimate is that one of 2820 persons has only atypical cholinesterase in serum (2). The purpose of the present work was to compare the activity of the esterase from such rare individuals with the activity of the normal esterase upon a series of *n*-aliphatic choline esters.

Methods

Hydrolysis of the choline esters was measured at $37.0 \pm 0.2°$ C in the Warburg apparatus using Krebs–Ringer solution of pH 7.4 as buffer and 5% CO_2 and 95% N_2 as the gas in the reaction vessels. Substrate was placed in the side arm with the enzyme occupying the main compartment of the vessel.

The manometers were read every half minute for 14 minutes when the concentration of substrate was low, and every minute for 28 minutes when the concentration of substrate was high. The rate of hydrolysis at each concentration of substrate was determined in duplicate, and each determination of a Michaelis constant (K_s) was based on measurements of enzyme activity at five or six concentrations of substrate spaced at 0.3 log unit intervals. Corrections were made for non-enzymic hydrolysis.

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[2]A part of the contents of this paper was presented in a thesis for the degree of Doctor of Philosophy in Pharmacology, University of Toronto, May 1958.

Serum concentrations were either 1 or 2% by volume for the normal sera, and 2 or 4% for the atypical sera. Blood was obtained by venipuncture and any serum samples containing haemolyzed cells were rejected. The majority of determinations were carried out using two individuals as the source of the normal type of pseudocholinesterase, and two individuals as the source of atypical pseudocholinesterase. There was no evidence of a systematic difference in K_s values between the two individuals with normal, or between the two with atypical pseudocholinesterase. However, some sample-to-sample variation in the apparent concentration of the esterase in the sera of any given person occurred regularly (5, 6). Adjustments were made for this by correcting the theoretical maximal velocity on the basis of determination of "pseudocholinesterase units" which were measured in the spectrophotometer with benzoylcholine as the substrate (6, 7); such an adjustment was justified since the correlation coefficient for the hydrolysis rates of acetylcholine vs. those of benzoylcholine was 0.97 for individual sera with various degrees of activity (7).

Of the substrates, acetylcholine chloride and benzoylcholine chloride were obtained commercially; propionylcholine *p*-toluene sulphonate and butyrylcholine *p*-toluene sulphonate were received as gifts.* The iodides of propionylcholine, butyrylcholine, pentanoylcholine, hexanoylcholine, and heptanoylcholine were synthesized in this laboratory according to the method of Kyi and Wilson (8) and all had the expected melting points. All compounds were stored over concentrated sulphuric acid in a vacuum desiccator. During the examination of each compound, the amounts of ester were repeatedly computed from the total volume of gas released during complete hydrolysis of a small sample.

The Michaelis constants (K_s) and maximal velocities (V_{max}) were calculated in the following manner. The observed volumes of gas released during hydrolysis were plotted against time and a line was fitted to the points by the eye. The slope of the line multiplied by the Warburg vessel constant was taken as the rate of hydrolysis, after correction for spontaneous hydrolysis of the substrate. The K_s and V_{max} values were then determined as means of the two linear transformations of Lineweaver and Burk (9). Another set of K_s and V_{max} values were obtained from the same data by plotting the observed rates of hydrolysis against the log concentration of substrate (Michaelis and Menten (10)). These values were then averaged with those from the Lineweaver–Burk determinations. The individual observations of K_s and V_{max} which made up these averages did not differ by more than 10 to 20% from one another. Each of these averages was then treated as a single value in the presentation of results.

The rates of hydrolysis of most esters were determined in 50-millimolar concentrations as a check on the calculated maximum velocities. In addition to the normal and atypical sera described above, purified pseudocholinesterase (Harvard fraction IV-6-3) was used. In order to obviate any corrections for fluctuations of activity, all determinations were performed on one sample of each normal, atypical, and purified esterase within as short a time as possible.

*Receipt of these compounds from Hoffman-LaRoche, Montreal, is gratefully acknowledged.

Each determination of rate was performed in duplicate on each of several days, and corrected for spontaneous hydrolysis.

Michaelis constants and maximum velocities for benzoylcholine were obtained by ultraviolet spectrophotometry, as described previously in detail (11). The method consists in recording continuous curves of the absorbance at 240 mμ at room temperature during the complete hydrolysis of the substrate.

Results

The K_s values (Michaelis constants) for normal and atypical pseudocholinesterase are presented in Table I. The theoretical maximal velocities (V_{max})

TABLE I

Michaelis constants (K_s) for *n*-acyl choline esters and the normal and the atypical pseudocholinesterases (K_s is given as mmoles per liter ± standard error, with the number of experiments in parentheses)

	Normal K_s	Atypical K_s	Ratio, atyp./normal
Acetylcholine Cl	1.40±0.04 (3)	9.0 ±0.10 (3)	6.42
Propionylcholine I	0.97±0.05 (2)	3.1 ±0.97 (3)	3.20
Butyrylcholine I	0.91 (1)	1.7 ±0.7 (2)	1.87
Pentanoylcholine I	0.72±0.04 (5)	1.5 ±0.17 (4)	2.09
Hexanoylcholine I	0.57±0.09 (3)	0.82±0.06 (3)	1.44
Heptanoylcholine I	0.38±0.22 (4)	1.11±0.14 (5)	2.92
Benzoylcholine Cl*	0.004±0.0003 (10)	0.022±0.003 (10)	5.5
Propionylcholine *p*-toluene SO_4	0.41±0.04 (4)	2.3 ±0.35 (3)	5.61
Butyrylcholine *p*-toluene SO_4	0.29±0.03 (6)	1.2 ±0.08 (3)	4.13

*Spectrophotometric determinations.

for both types of pseudocholinesterase are given in Table II. Butyrylcholine iodide was hydrolyzed most rapidly of all substrates by the normal enzyme. The data suggest that pentanoylcholine is the optimal substrate of atypical

TABLE II

Maximal velocities (V_{max}) calculated for the hydrolysis of *n*-acyl choline esters by the normal and the atypical pseudocholinesterases (V_{max} is given as μmoles of ester hydrolyzed per ml of serum per minute ± standard error, with the number of experiments in parentheses)

	Normal V_{max}	Atypical V_{max}	Ratio, atyp./normal
Acetylcholine Cl	4.21±0.20 (4)	1.30±0.12 (4)	0.310
Propionylcholine I	6.91±0.2 (2)	1.67±0.30 (3)	0.242
Butyrylcholine I	10.1 (1)	2.25±0.02 (2)	0.222
Pentanoylcholine I	7.99±0.32 (5)	2.77±0.02 (4)	0.347
Hexanoylcholine I	4.58±0.21 (3)	1.33±0.03 (3)	0.290
Heptanoylcholine I	4.41±0.22 (4)	1.62±0.05 (5)	0.367
Benzoylcholine Cl*	1.9 ±0.1 (10)	0.99±0.08 (10)	0.5
Propionylcholine *p*-toluene SO_4	5.39±0.38 (4)	1.34±0.22 (3)	0.249
Butyrylcholine *p*-toluene SO_4	6.52±0.14 (6)	1.88±0.16 (3)	0.289

*Spectrophotometric determinations.

esterase; however, on account of additional data given below, this result must be viewed with the reservation that the hydrolysis rates of the iodides of

butyryl and pentanoylcholine by atypical esterase are not significantly different in relation to the over-all standard error of the tabulated data. Table II does not permit the assumption that atypical esterase hydrolyzes butyrylcholine faster than pentanoylcholine.

In an effort to check the calculated maximum reaction velocities in an experiment of simple design, several cholinester iodides were tested as substrates in 50-millimolar concentrations. The relative reaction rates by crude normal serum and by Harvard fraction IV-6-3 (cholase) were similar (Table III). The normal enzyme hydrolyzed butyrylcholine iodide faster than

TABLE III

Rates of hydrolysis of choline ester halides
Summary of data, expressed in per cent of pertinent rates of hydrolysis of butyrylcholine

Substrates	Maximum velocities (cf. Table II)		Observed velocities with 50-m*M* substrate concentrations		
	Normal serum	Atypical serum	Cholase	Normal serum	Atypical serum
Acetylcholine	42	58	54	54	38*
Propionylcholine	68	74	—	—	—
Butyrylcholine	100	100	100	100	100
Pentanoylcholine	79	121	90	86	98
Hexanoylcholine	45	59	39	38	61
Heptanoylcholine	44	72	31	37	65

*Substrate concentration insufficient to saturate the enzyme.

it did pentanoylcholine iodide but the atypical enzyme hydrolyzed the two esters at the same rate. An analysis of variance showed a significant interaction term which indicated that the relative reaction rates of the two enzymes differed for the substrates butyryl-, pentanoyl-, and hexanoyl-choline. Simple dilution as a cause of the difference between enzymes is thereby precluded.

Discussion

Within experimental error (Table III), the purified esterase (Harvard fraction IV-6-3) and crude normal serum pseudocholinesterase hydrolyzed the esters studied at the same relative rates. This implied that the substrates were hydrolyzed by the same enzyme and confirms a recent report (12). Furthermore, within the range of experimental error, no deviations from the theoretical sigmoid curve (10) or its linear transformation (9) were encountered for either the normal or atypical serum pseudocholinesterase, although such deviations have occasionally been described in the literature (13, 14, 15).

The Michaelis constant for every substrate investigated was greater with the atypical than with the normal enzyme (Table I). In other words, the atypical esterase had a decreased apparent affinity for all choline esters reported here; in addition, decreased affinities of this enzyme have been observed for benzoylcholine (16), succinylcholine (17), procaine (Kalow, unpublished results; cf. ref. 3), and for numerous inhibitors (3). This has, of course, initially aroused suspicion that the difference between sera with normal and atypical cholinesterase activity is caused by the presence of a naturally

occurring, unknown, competitive inhibitor in the atypical sera. However, this suspicion has not found experimental support (4).

Confining attention to the data obtained with halide salts of the choline esters (Table I), the K_s values decrease with increasing length of the acyl chain for both enzymes. If log K_s is plotted versus the number of acyl carbons in the series from acetylcholine to hexanoylcholine, the two factors seem to be linearly related (Fig. 2). However, the data for the atypical enzyme do not fit the

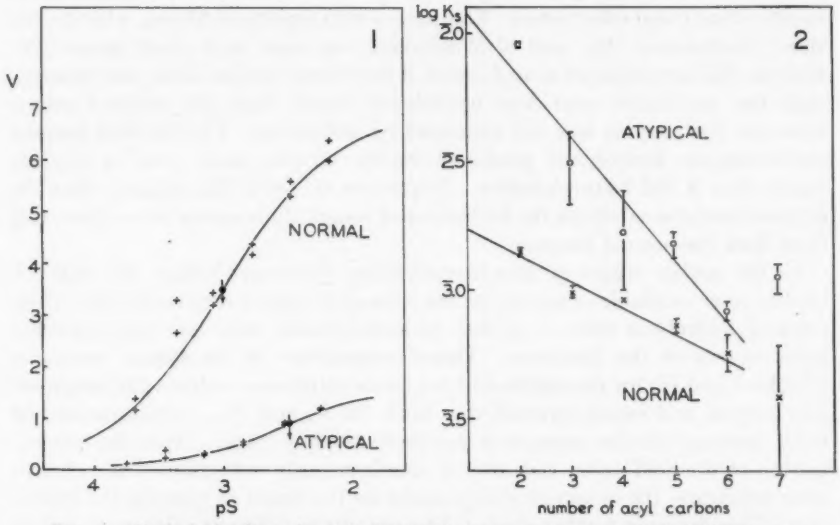

FIG. 1. Hydrolysis of propionylcholine iodide by normal and atypical cholinesterases. Abscissa: negative log of molar concentration of substrate. Ordinate: reaction velocities in arbitrary units. The arrows indicate the Michaelis constants.

FIG. 2. Michaelis constants (K_s) of a series of n-acyl choline esters (halide salts) and normal and atypical cholinesterase of human serum. The vertical lines indicate the standard errors of the means. The calculated sloped lines indicate the relationship $\log K_s = 0.675-0.243n$ for atypical esterase, and $\log K_s = 1.662-0.101n$ for normal esterase, where n equals the number of acyl carbons. These equations have been calculated from all data except those obtained with heptanoylcholine; no linear relationship between n and $\log K_s$ can be assumed to exist for atypical esterase if the latter data are included.

assumption of a linear relationship if the plot is extended to include heptanoylcholine. The straight lines shown in Fig. 2 have the slopes -0.24 ± 0.06 for atypical, and -0.10 ± 0.01 for the normal esterase. The difference between the slopes is statistically significant ($p < 0.01$). Bergmann and Segal (18) found slopes of about -0.3 for pseudocholinesterases (presumably normal type) and two homologous series of inhibitors when pI_{50} was plotted against chain lengths. Bergmann calculated that this slope was proportional to the free energy change (slope $\times$ $2.303RT = \Delta\Delta F$) associated with the transfer of one $—CH_2$ group from the solution to the state of adsorption on the enzyme surface. The difference between the slopes reported by Bergmann and those of Fig. 2 may indicate a fundamental difference between I_{50} and the more complicated K_s.

Data obtained with a series of inhibitors suggested that, as a rule, the more potent an inhibitor the greater the difference of its affinities for the normal and atypical cholinesterase (3). This rule seems to be reversed for the substrates described here. However, the apparent affinities of the inhibitors for the normal esterase varied over a 20-millionfold range, whereas the apparent affinities within this homologous series of substrates vary over only a fivefold range. Hence, the trend described here may be a subordinate one.

The normal serum pseudocholinesterase hydrolyzed butyrylcholine more rapidly than it did other esters. This agrees with reports of Myers, who studied three cholinesters (22), and of Whittaker, who used acyl–alkyl esters (23). Hofstee (12) investigated *n*-acyl esters of *m*-hydroxybenzoic acid, and reported that the pentanoyl ester was hydrolyzed faster than the butyryl esters; however, the enzyme was not saturated by substrates. The atypical pseudocholinesterase hydrolyzed pentanoylcholine at the same rate or slightly faster than it did butyrylcholine. Inspection of Table III suggests that the atypical enzyme catalyzes the hydrolysis of longer chain esters more effectively than does the normal enzyme.

In the earlier stages of this investigation, propionylcholine and butyrylcholine were available to us only as the salts of *p*-toluene sulphonic acid. Their rates of hydrolysis relative to that of acetylcholine were less than expected from reports of the literature. Direct comparison of the kinetic constants (Tables I and II) for the iodide and *p*-toluene sulphonate salts of the propionic and butyric acid esters revealed that both the K_s and V_{max} constants seemed to be decreased in the presence of *p*-toluene sulphonic acid. Since the concentration of the acid anion was altered simultaneously with that of the choline ester substrate, the observed effects might be the result of competitive inhibition. This deserves further study. The use of the different salts may explain the apparent discrepancy between the relative rates of hydrolysis of butyrylcholine and acetylcholine reported by Myers (20) and Whittaker (21), and those reported by Wilson (13).

Acknowledgments

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References

1. W. KALOW and N. STARON. Can. J. Biochem. and Physiol. **35**, 1305 (1957).
2. W. KALOW and D. R. GUNN. Ann. Human Genet. **23**, 239 (1959).
3. W. KALOW and R. O. DAVIES. Biochem. Pharmacol. **1**, 183 (1958).
4. W. KALOW. *In* Ciba Foundation Symposium on Human Biochemical Genetics in Relation to the Problem of Gene Action. London. 1959.
5. K.-B. AUGUSTINSSON. Acta Physiol. Scand. **35**, 40 (1955).
6. H. A. LINDSAY. Ph.D Thesis, University of Toronto, Toronto, Ont. 1956.
7. W. KALOW and H. A. LINDSAY. Can. J. Biochem. and Physiol. **33**, 568 (1955).
8. S. Y. KYI and W. WILSON. Brit. J. Pharmacol. **12**, 38 (1957).
9. H. LINEWEAVER and D. BURK. J. Am. Chem. Soc. **56**, 658 (1934).
10. L. MICHAELIS and M. L. MENTEN. Biochem. Z. **49**, 333 (1913).
11. W. KALOW, K. GENEST, and N. STARON. Can. J. Biochem. and Physiol. **34**, 637 (1956).
12. B. H. J. HOFSTEE. J. Pharmacol. Exptl. Therap. **123**, 108 (1958).
13. I. B. WILSON. J. Biol. Chem. **208**, 123 (1954).
14. E. HEILBRONN. Acta Chem. Scand. **12**, 1879 (1958).

15. W. HARDEGG, D. BECHINGER, and R. DOHRMANN. Pflüger's Arch. **263**, 33 (1956).
16. W. KALOW. Can. Anaesthetists' Soc. J. **3**, 22 (1956).
17. W. KALOW. Anesthesiology, **20**, 505 (1959).
18. F. BERGMANN and R. SEGAL. Biochem. J. **58**, 692 (1954).
19. G. E. BRIGGS and J. B. S. HALDANE. Biochem. J. **19**, 338 (1925).
20. D. K. MYERS. Biochem. J. **55**, 67 (1953).
21. V. P. WHITTAKER. Physiol. Revs. **31**, 312 (1951).

BIOCHEMICAL STUDIES ON SOCKEYE SALMON DURING SPAWNING MIGRATION

XII. LIVER GLYCOGEN[1]

VIOLET M. CHANG AND D. R. IDLER

Abstract

Liver glycogen levels were determined for a pure stock of sockeye salmon (*Oncorhynchus nerka*) taken at three locations during spawning migration. The liver glycogen content of the male was found to be consistently greater than that of the female throughout the entire river migration. In both sexes liver glycogen decreased during the earlier phase of migration, but increased during the later stage so that the levels at the spawning grounds were approximately twice those at the mouth of the river. The changes which occur are discussed in relation to sex differences, energy expenditures, and plasma steroid hormone levels.

Introduction

During the 1957 Fraser River sockeye salmon run to Stuart Lake, an extensive program was initiated at this Station to investigate the biochemical changes which occur during spawning migration (1). As part of this program the liver glycogen content at various stages of the river migration of the 1959 Chilko Lake migrants was studied and the results are presented in this paper.

Materials and Methods

The liver samples were obtained from the 1959 Chilko Lake sockeye salmon migrants at Albion (situated near the mouth of the Fraser River), at Siwash Bridge (358 miles upstream from Albion), and at the spawning grounds on the Chilcotin River. The fish at Albion were caught in a gill net and transferred to a "live" box which was anchored beside the boat. The fish were processed as quickly as possible. A dip net was used to capture fish at Siwash Bridge and a reef net on the spawning grounds. After the fish had been bled by severing the caudal artery, the livers were removed, immediately frozen in liquid nitrogen, and stored in separate polyethylene bags on dry ice. The frozen liver samples were individually powdered in a mortar with liquid nitrogen. Aliquots of approximately 10 g were placed in 100-ml beakers covered with aluminum foil and stored on dry ice until analyzed.

The livers were homogenized with trichloracetic acid (TCA) in a Servall omnimixer at a speed of 14,000 r.p.m. The first extraction was carried out with 30 ml of 10% TCA for 5 minutes, after which the mixture was centrifuged at 12,000 r.p.m. for 15 minutes. The supernatant fluid was decanted and the residue was extracted twice more with 30 ml of 5% TCA for 2 minutes. The supernatants from all extracts were combined and diluted to 100 ml. The procedure was carried out at 0° C.

The glycogen was precipitated by mixing 1 volume of TCA extract with 5 volumes of absolute ethanol in a stoppered conical centrifuge tube and

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refrigerating overnight. The precipitated glycogen was collected by centrifugation at 3000 r.p.m. for 15 minutes. The supernatant was discarded and the tubes were allowed to drain in an inverted position for at least 10 minutes. The residue was dissolved in a measured quantity of distilled water.

Glycogen was assayed with the anthrone reagent. The color was developed according to Carroll *et al.* (2) and read in an Evelyn colorimeter using a 620 mμ filter.

Results

The results of liver glycogen determinations on both male and female fish at various stages of spawning migration are shown in Table I.

TABLE I

Liver glycogen (mg/g liver (wet weight)) in migrating sockeye salmon at three locations

Albion		Siwash Bridge		Chilcotin River		
				Postspawned		Prespawned†
M	F	M	F	M	F	M
22.2*(1a)	2.57(1a)	1.15(2a)	0.83(2a)	21.9	2.31	45.5
19.1*(1a)	0.87(1a)	9.89(2a)	1.20	35.5	3.61	68.7
16.1	0.36*	7.93(2a)	1.58(2a)	41.8	4.40	33.4
11.1*	1.97*	4.76(2a)	2.52(2a)	63.1	3.90	54.1
15.1	0.40*	7.74	3.68	11.5	8.34	26.7
24.3*(1b)	4.93*(1b)	6.90	2.23(2b)	39.8	6.28	55.1
21.6*(1b)	3.45(1b)	7.98	1.20	8.6	0.21	63.7
16.8*(1b)	1.61*(1b)	6.63(2b)	1.54(2b)	39.6	7.12	18.0
12.0*	3.69*(1b)	5.84(2b)	1.88(2b)	35.4	7.56	36.9
9.7*	—	9.04(2b)	—	23.0	—	50.3
27.4	—	—	—	—	—	—
17.8±1.7‡	2.20±0.53	6.79±0.78	1.85±0.29	32.0±5.1	4.37±0.93	45.2±1.62

*Scale readings showed these samples were from Chilko Lake fish. All fish taken at Siwash Bridge and the Chilcotin River were Chilko Lake fish.

†These are presented as minimal values.

1. Samples obtained Aug. 6, 1959. (*a*) Analyses completed Aug. 12, 1959. (*b*) Analyses completed Aug. 19, 1959.
2. Samples obtained Aug. 29, 1959. (*a*) Analyses completed Sept. 10, 1959. (*b*) Analyses completed Oct. 26, 1959. These data indicate that no detectable loss of liver glycogen occurred in samples which had been stored up to 8 weeks. In almost all cases analyses were completed on samples within 4 weeks after they had been obtained.

‡Mean ± standard error.

NOTE: M = male; F = female.

It is probable that the variation of liver glycogen content in individuals was attributable in part to the degree of muscular exhaustion due to struggling of the fish during capture. However, Miller *et al.* (3) have determined in experiments with hatchery-reared rainbow trout that the liver glycogen of well-nourished fish was relatively stable to change during exercise even though muscle glycogen was depleted. The level of liver glycogen in the individual fish may therefore be regarded as an indication of its physiological condition and nutritional state as compared with others sampled at the same location. In regard to the samples taken at the spawning grounds, no attempt was made to differentiate between fish which had just spawned and ones for which a time lapse had occurred after completion of spawning.

In spite of the variation in liver glycogen between individual fish of the same sex sampled at the same location, an over-all trend was seen in relation to the

levels of glycogen in the two sexes. The most striking feature of the data in Table I is the large and consistent difference between both sexes at all three points. The mean glycogen level of the male liver was 8 times more at Albion, 3.7 times more at Siwash Bridge, and 7.3 times more at the spawning grounds than that of the female.

Whereas the liver glycogen of the male decreased 61.8% from Albion to Siwash Bridge, the loss from the female was only 16%. From Siwash Bridge to the spawning grounds these levels increased so that values in postspawned fish were 1.8 times higher for the male and 1.98 times higher for the female than those found at Albion.

Unfortunately, the samples obtained from "prespawned" salmon on the Chilcotin River experienced a short period of thawing before they were analyzed (the dry ice evaporated during the night and the temperature outside the box was +3° C). Consequently, the data for these samples have been presented as minimal values only and were included to indicate the appreciably higher levels which may have been contained in these samples since a loss in liver glycogen was found in the Albion samples which had undergone the same period of thawing when compared with samples that had been analyzed prior to being thawed. Table II shows the effect of thawing on the Albion samples.

TABLE II

Glycogen content of Albion sockeye livers before and after thawing

Sex	Before, mg/g*	After, mg/g*	Decrease due to thawing, %†
M	22.17	14.25	35.7
M	11.99	3.74	68.9
M	2.73	0.40	85.4
F	0.517	None	100
F	3.69	0.31	91.6

*Based on the wet weight of the liver.
†These samples were analyzed prior to thawing and stored in the same box with the samples from prespawned fish which were analyzed after thawing.

Discussion

The liver glycogen values, and the variation for individual fish obtained in this study, are similar to those reported by Fontaine and Hatey (5) for the Atlantic salmon, *Salmo salar*, with respect to the fish captured during the winter at the spawning grounds. On the other hand, Greene (6) found lower liver glycogen levels in the king salmon, *Oncorhynchus tschawytscha*, whose average values at the beginning and end of migration were only 0.405% and 0.057% respectively. Another difference was that a decrease occurred in the liver glycogen content of the king salmon during reproductive migration, but an increase in content was found in the Chilko Lake sockeye migrant.

It is of interest to note that the liver glycogen content of males at the Chilcotin River spawning grounds (4.5%) fell within the range found for normal human liver (0.31–5.98% with a mean of 2.2%) (7) and compared with that of the rat (2.5–8.3%) and dog (6.1%) (8).

The sex difference in hepatic glycogen content in normal fasting and pregnant mammals has been described (9, 10, 11, 12). This inequality of liver glycogen in the two sexes was found during spawning migration of *Salmo salar* (5) and the observations of these workers have been confirmed in the present study. The smaller glycogen deposition in the liver of the female as compared to that in the male may be associated with the greater increase in the weight of the ovaries (1) and the accumulation of carbohydrates in the ovaries. A glucose content of 0.096% (6) and a glycogen content of 0.119% (5) have been reported for roe. Fontaine and Hatey (5) found that the glycogen content of the testes of the Atlantic salmon was only 0.017%. Furthermore, the smaller amount of liver glycogen in the female may be related to a greater estrogenic hormone level produced during the rapid sexual development associated with spawning migration. The depressant effect on liver glycogen levels following administration of natural estrogens has been demonstrated with male rabbits (13) and with rats (10). This effect of a hypersecretion of hormones, associated with the reproductive cycle, on liver glycogen deposition during spawning migration is suggested when the results obtained on *Salmo salar* (5), captured in the spring at the beginning of river migration, are compared to those of the same fish at the spawning grounds in the winter. The spawning migration of *Salmo salar* takes place over a period of many months and involves a slow and gradual development of the gonads. On the other hand, Chilko Lake sockeye spend only 36 days between Albion and the completion of the spawning act (14). Therefore, the results obtained by Fontaine and Hatey at the beginning of river migration have no chronological equivalent for most Pacific sockeye since the sockeye would be feeding before its entrance into fresh water. A run of sockeye to Cultus Lake, B.C., probably approximates most the long residence of *Salmo salar* in fresh water.[2]

Although the average liver glycogen value for the postspawning female on the Chilcotin River was 1.98 times greater than that at Albion, the variations in the individual samples were too great for the mean values to be very significant ($p > .05$). This increase was statistically significant for the male. These results may be interpreted in relation to energy expenditure studies which were carried out on the 1956 Chilko Lake run. In these considerations the two locations, Siwash Bridge and Keighley Holes, are only 18 miles apart and may be regarded as comparable points. It takes the fish 17 days to travel from Albion to Siwash Bridge and spawning begins 16–17 days later. Spawning is completed in 2–3 days.

From the fat and protein expenditure studies (14), in terms of kilogram-calories expended per day of travel, the standard male fish used 68.4 Cal/day of fat and 0.313 Cal/day of body protein in travelling from Albion to Keighley Holes, while the standard female fish consumed 87.5 Cal/day of fat and 10.1 Cal/day of protein for the same distance. From Keighley Holes to the completion of spawning the amount of fat expended by the standard male was 67.3 Cal/day and for the standard female was 68.5 Cal/day. At the same time,

[2]Personal communication from S. R. Killick of the International Pacific Salmon Fisheries Commission.

protein consumption increased in both sexes, with the standard male using 27.7 Cal/day and the standard female 31.8 Cal/day. Thus, fat was the principal source of energy between Albion and Keighley Holes (Siwash Bridge) and during this period there was a decrease in liver glycogen. From Siwash Bridge to Chilko Lake a change from predominantly fat metabolism to an increased protein metabolism was coincident with an increase in liver glycogen between these two points. This finding is consistent with that for normal fasting animals (15) where an initial decrease is followed by a rise in liver glycogen which is accompanied by a parallel increase in urinary nitrogen excretion of a magnitude sufficient to account for the extra carbohydrate in terms of increased protein catabolism.

Although gluconeogenesis from non-carbohydrate sources occurs in normal fasted animals to replenish the depleted liver glycogen somewhat, this level is usually far below that encountered in the non-fasting state (15). However, with injections of cortical extracts, and especially those with the oxygen function at C-11 and C-17 (16, 17), liver glycogen levels during fasting are usually increased to equal or to approach those of the non-fasting animals. It has been reported that the adrenal cortical steroids do not influence the rate of glucose utilization but increase the production of glucose by increased gluconeogenesis. Rosen *et al.* (18) have found that glucocorticoids markedly stimulate in liver the activity of glutamic–pyruvic transaminase, which they propose as the enzymatic basis in gluconeogenesis. Recently, cortisone and cortisol were found in high concentration in the plasma of migrating sockeye salmon, and the levels at the spawning ground were much greater than those at the beginning of migration (4). Therefore, the appearance of elevated cortical steroid levels together with the metabolism of body protein may be regarded as factors which contribute to the increased levels in liver glycogen as the fish approach the spawning grounds.

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References

1. D. R. IDLER and H. TSUYUKI. Can. J. Biochem. and Physiol. **36**, 783 (1958).
2. N. V. CARROLL, R. W. LONGLEY, and J. H. ROE. J. Biol. Chem. **220**, 583 (1956).
3. R. B. MILLER, A. C. SINCLAIR, and P. W. HOCHACHKA. J. Fisheries Research Board Can. **16**, 321 (1959).
4. D. R. IDLER, A. P. RONALD, and P. J. SCHMIDT. Can. J. Biochem. and Physiol. **37**, 1227 (1959).
5. M. FONTAINE and J. HATEY. Physiol. Comparata et Oecol. **3**, 37 (1952).
6. C. W. GREENE. J. Biol. Chem. **48**, 429 (1921).
7. J. E. SOKAL and K. E. GERSZI. J. Lab. Clin. Med. **53**, 876 (1959).
8. E. S. WEST and W. R. TODD. Textbook of biochemistry. The Macmillan Co., Toronto. 1955. p. 944.
9. H. J. DEUEL, JR., M. GULICK, C. F. GRUNEWALD, and C. H. CUTLER. J. Biol. Chem. **104**, 519 (1934).
10. M. GULICK, L. T. SAMUELS, and H. J. DEUEL, JR. J. Biol. Chem. **105**, 29 (1934).
11. H. J. DEUEL, JR., J. S. BUTTS, L. F. HALLMANN, S. MURRAY, and H. BLUNDEN. J. Biol. Chem. **119**, 617 (1937).

12. L. CHEDID, F. BOYER, and M. SAVIARD. Ann. endocrinol. **16**, 472 (1955).
13. E. STARK and C. NIKODEMUSZ. Z. Vitamin-, Hormon- u. Fermentforsch. **4**, 221 (1951).
14. D. R. IDLER and W. A. CLEMENS. International Pacific Salmon Fisheries Commission. Progr. Rept. 1955. p. 75.
15. C. N. H. LONG, B. KATZIN, and E. G. FRY. Endocrinology, **26**, 309 (1940).
16. C. N. H. LONG. Endocrinology, **30**, 870 (1942).
17. S. W. BRITTON and E. L. CORRY. Am. J. Physiol. **131**, 790 (1941).
18. F. ROSEN, N. R. ROBERTS, L. E. BUDNICK, and C. A. NICHOL. Science, **127**, 287 (1958).

BIOSYNTHESIS OF QUERCETIN IN BUCKWHEAT

PART III[1]

J. E. WATKIN AND A. C. NEISH

Abstract

The effect on the conversion of carbon[14] to quercetin by a buckwheat plant caused by variation in the dose rate of a carbon[14]-labelled precursor in μmoles over a 100-fold range has been studied. The results expressed as dilution of carbon[14] varied over a 200-fold range for the same precursor with most of the variation being found at low dose rates. When expressed as the percentage of administered carbon[14] converted to quercetin the results for acetate, D-glucose, and L-phenyllactic acid were mostly constant over this range but L-phenylalanine had a maximum percentage conversion or an optimal dose rate. An explanation of the results in metabolic terms has been attempted.

It is recommended that the "percentage of C^{14} converted" be used to express results in plant biosynthetic work.

Introduction

Radio tracer techniques are being widely used for the investigation of biosynthetic pathways in plants In many of these an arbitrarily chosen amount of the C^{14}-labelled compound is introduced into a plant, or portion of a plant, and after a period of time the incorporation of C^{14} into selected plant products is measured. This general procedure was used in previous papers of this series (1, 2) as well as in other studies in this laboratory in the biosynthesis of lignin (3, 4, 5) and simple aromatic compounds (7, 8).

Where it is desirable to compare several labelled compounds for their relative efficiency as precursors, a decision has to be made on the dose of each to be fed to the plant. It might be thought that the optimal amount of the precursor to feed is the smallest amount that would give measurable activity in the product isolated. This small amount will vary with the specific activity of the compound concerned. Some C^{14} compounds are obtained by multistage synthesis and it is not practicable to obtain them with high specific activity. Large doses (which may be above the level at which plant metabolism is unaffected) are then required to produce measurable activity in the end product. Other compounds can only be fed at low dosages because of their low solubility, e.g. tyrosine. It is difficult therefore to compare a series of compounds at the same dose level. Any level set would be arbitrary and difficult to achieve. In the present experiment, where single plants were used, there was considerable natural variation in size of each plant and on feeding fixed amounts of precursor further variation of the dose rate was introduced. Also it may not be fair to compare compounds with widely different structures at the same dose level, and, further, some compounds may have an optimum dose rate.

For these reasons it has not been possible to keep the dose rate constant and there was no basis for quantitative comparison between experiments using

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different precursors at varying dose rates and different batches of plants. The effect of varying the dosage (i.e. μmoles of precursor fed per gram of dry weight of plant) of several precursors was investigated in the biosynthesis of quercetin by buckwheat. The results are reported in this paper.

A study on the effect of variation in the dose of acetate on rat liver slices has been reported by Emerson and Van Bruggen (9), who found that the dose affected the different metabolic pathways of acetate to a greatly varying extent.

Experimental

Buckwheat (*Fagopyrum tataricum* Gaertn. var. C.D. 4251) was grown in a growth chamber under fluorescent lights and at a controlled temperature. When the plants were about four weeks old and weighed about 30 grams the roots were cut off under water and the cut end of the shoot put in a solution of radioactive precursor. After a period of time for metabolism the plant was blended in hot 80% ethanol (2). The glycoside rutin was isolated from the extract by adsorption on Magnesol (10). The rutin was then hydrolyzed to quercetin and sugars and the activity in the quercetin measured after conversion to the pure pentaacetate (1).

Determination of Natural Precursors

The 80% ethanol extract of buckwheat was neutralized, extracted with petroleum ether (b.p. 100–120° C) to remove chlorophyll, and evaporated to small volume under reduced pressure. An aliquot was extracted with *n*-butanol once, adjusted to pH 4, and steam distilled in a micro-Kjeldahl apparatus, the volatile acid being titrated to give the free acetic acid content (11). The free phenylalanine in the extract was adsorbed on Dowex I acetate resin, eluted with 0.5 *N* acetic acid, and estimated colorimetrically with ninhydrin (12). The deionized extract was concentrated and applied to a charcoal–Celite column and the sugars, glucose, fructose, and sucrose obtained by elution with aqueous ethanol. The sugars were weighed and the total weight expressed as μmoles of monosaccharide.

Results

Table I shows the effect of time on the incorporation of an approximately constant dose of L-phenylalanine-C^{14} into quercetin. The L-phenylalanine was all adsorbed during the first ½ hour. The percentage of C^{14} incorporated was relatively constant over a period of 8 to 24 hours.

The effect of varying the administered dose on incorporation of C^{14} into quercetin is shown in Table II. Several precursors which had been previously shown (1, 2) to be converted to quercetin were tested. A period of 24 hours was allowed in these experiments.

The first five columns of figures in Table II contain the experimentally determined data and the other figures are calculated from them. Most of these calculations are explained in the footnotes to the table but the method of calculating the dilution values depended on the precursor fed. The dilution values were calculated on a molar basis and represent the number of times the

TABLE I

Rate of incorporation of carbon^{14} from L-phenylalanine-C^{14} into quercetin

	Duration of experiment, hours					
	2	4	8	12	24	48
L-Phenylalanine fed, μmoles	15	15	15	15	15	15
C^{14} fed, μc	5.05	5.05	5.05	5.05	5.05	5.05
Dry wt. of plant, g	4.2	3.0	4.2	4.1	2.1	2.6
Rutin isolated, mg	85	64	87	81	49	57
Specific activity of quercetin, mμc/μmole	0.88	1.44	1.32	1.29	2.41	1.30
Total C^{14} in quercetin, mμc	123	153	188	171	194	122
Dose rate, μmoles/g dry wt.	3.6	5.0	3.6	3.7	7.1	5.8
% C^{14} fed in quercetin	2.4	3.0	3.7	3.4	3.8	2.4
Dilution value*	380	234	255	261	140	259

*See text under "Results" for calculation of dilution values.

activity in a mole of given precursor was diluted on conversion into quercetin. Since 1 mole of a labelled C_6–C_3 compound is used for synthesis of 1 mole of quercetin (1), the six A-ring carbons being inactive, the dilution values for the phenylpropanoid compounds were calculated by dividing the specific activity of the precursor (mμc per μmole) by that of quercetin. As 3 moles of labelled acetate are used for the biosynthesis of 1 mole of quercetin (2) (forming the A ring, the remaining nine carbons being inactive) the molar specific activity of the quercetin was divided by 3, before this calculation was made, in the acetate-feeding experiments. The 15 carbons of quercetin are derived equally from glucose (2). Since 15/6 moles of glucose are required to supply the carbon for 1 mole of quercetin, the molar specific activity of quercetin was divided by 2.5 in calculating the dilution values when glucose-C^{14} was fed.

The results in Table II are arranged in order of increasing dosage for each group of experiments. The first group of phenylalanine-feeding experiments was done with plants grown and treated at the same time. This is also true of the glucose and acetate experiments. The second group of phenylalanine experiments and the phenyllactic acid and cinnamic acid experiments were carried out with plants grown at different times.

The most striking result in Table II is the large change in the dilution value with variation in the dosage. This is especially noticeable where low dose rates were used, the rate of change of dilution value with dose being very large for doses of phenylalanine below 5 μmole/g and for doses of glucose and acetate below 20 μmole/g.

The percentage of C^{14} in phenylalanine converted to quercetin (column 9, Table II) passes through a maximum as the dose rate is varied. There are also maxima for acetate, glucose, and phenyllactic acid but they are much less marked than for phenylalanine and could fall within experimental error. In general the changes in percentage of C^{14} converted are quite small compared with the large changes in the dilution values as the dose varied. The results for the first group of phenylalanine experiments are plotted in Fig. 1.

TABLE II

The effect of variation in the administered dose of precursor on its conversion to quercetin*

Precursor	Amount fed		Dry wt. of plant, g	Wt. of rutin, mg	Sp. act. of quercetin, mμc/μmole	C^{14} in† quercetin, mμc	Dosage of precursor, μmole/g dry wt.	Dilution§ value	% of C^{14}‡ converted	μmole precursor¶ converted
	μmole	μc								
L-Phenylalanine-G-C^{14}	1	2.7	2.8	67	0.47	52	0.36	5,700	1.9	—
	4	10.9	3.0	79	4.2	540	1.3	640	5.0	—
	10	13.6	2.1	54	15.1	1340	4.8	90	9.8	—
	20	13.6	3.1	80	7.7	1010	6.5	88	7.4	—
	50	13.6	3.5	90	5.3	780	14	51	5.7	.80
	100	8.2	2.0	50	4.2	350	50	20	4.3	2.2
L-Phenylalanine-G-C^{14}	15	5	3.8	88	1.3	190	4	260	3.8	—
	15	5	2.1	48	2.4	190	7	140	3.8	—
	16	4.2	2.1	78	2.5	320	8	105	7.6	—
	15	5.15	1.7	24	7.9	310	9	44	6.0	—
	62	5	6.1	145	1.6	380	10	50	7.6	—
	35	2.15	3.1	106	1.3	230	11	47	10.5	1.2
	46	10	3.0	72	5.5	650	15	40	6.5	1.0
	56	3.5	3.2	75	0.96	120	18	65	3.4	.6
	37	2.3	1.9	29	3.4	160	20	18	7.0	1.4
	37	2.3	1.6	27	2.3	100	23	27	4.4	1.0
D-Glucose-G-C^{14}	8	4.2	3.7	100	0.093	15	2.2	14,100	0.36	—
	20	4.2	4.6	125	0.075	15	4.4	7,000	0.36	—
	40	4.2	3.6	98	0.26	41	11	1,020	1.0	1.7
	100	4.2	3.5	101	0.27	45	29	380	1.07	1.7
	200	4.2	3.8	106	0.18	31	53	290	0.74	1.6
	400	4.2	3.2	89	0.24	35	125	110	0.83	2.3
Acetic acid-1-C^{14}	8	11.85	4.3	106	0.92	160	2	4,800	1.35	—
	20	11.85	4.3	100	1.03	170	5	1,700	1.43	—
	40	11.85	6.0	147	0.69	170	7	1,300	1.43	—
	100	18.2	4.7	117	1.75	340	21	300	1.87	—
	200	17.6	4.5	114	1.87	350	45	140	2.0	.9
	400	17.2	4.2	103	1.32	220	95	98	1.28	1.2

TABLE II (*Concluded*)

The effect of variation in the administered dose of precursor on its conversion to quercetin* (*Concluded*)

Precursor	Amount fed		Dry wt. of plant, g	Wt. of rutin, mg	Sp. act. of quercetin, mμc/μmole	C^{14} in† quercetin, mμc	Dosage of precursor, μmole/g dry wt.	Dilution§ value	% of C^{14}‡ converted	μmole precursor¶ converted
	μmole	μc								
L-Phenyllactic acid (Ring+β-C^{14})	3	0.315	2.9	72	0.068	8	1	1,500	2.6	—
	15	1.60	2.2	42	0.71	49	7	150	3.1	—
	24	2.5	3.0	74	0.90	110	8	116	4.4	—
	58	6.0	4.0	89	1.2	175	15	86	2.9	—
Cinnamic acid	14	8.5	2.5	42	0.3	21	6	2,000	0.25	—
	58	5.36	1.0	20	0.55	18	58	170	0.34	—
	86	51.4	.75	7	15	172	115	40	0.34	—

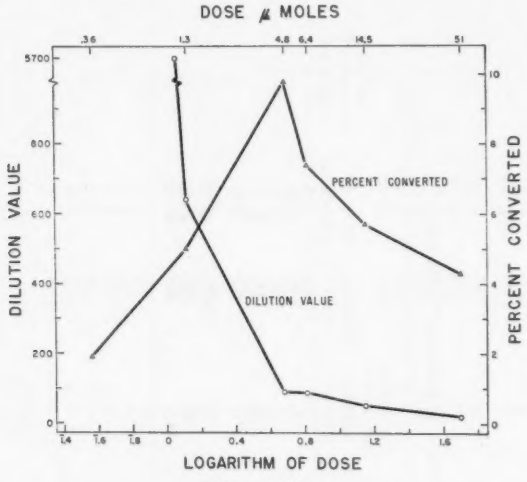
*The metabolic period was 24 hours.

†Total mμc of C^{14} in quercetin = μmoles of rutin × mμc per μmole of quercetin.

§See text under "Results" for calculation of dilution values.

‡% of C^{14} converted = $\frac{\text{total m}\mu\text{c in quercetin} \times 100}{\text{m}\mu\text{c fed}}$.

¶μmole of precursor converted = % of C^{14} converted × (μmole of precursor fed + μmole endogenous precursor).



Discussion

It is not possible to give a quantitative interpretation of the results obtained for such a complex system. However, it is possible to obtain a concept of why the dilution values vary so much with the dose rate while the percentage conversion figures remain relatively constant. Dilution of a C^{14}-labelled precursor in quercetin synthesis may be caused by (*a*) dilution in the endogenous pool of precursor, (*b*) dilution by quercetin formed from unlabelled precursors during the experiment, and (*c*) dilution by preformed quercetin. The dilution by preformed quercetin is not related to the dose rates but the other two factors which cause dilution would be expected to be.

Dilution in the endogenous pool may be considered first. This pool consists of any of the precursor of quercetin which is naturally present in the plant as well as any other compounds which equilibrate with it at a rate which is rapid compared to the rate of quercetin synthesis. Although it may be a simple matter to determine the amount of a substance naturally present it is difficult to assess quantitatively the substances which equilibrate with it.

The free phenylalanine pool in buckwheat at the stage of growth used was about 5 μmoles per gram dry weight. The smallest amount of phenylalanine fed would be diluted about 15 times by this pool while the largest dose would only be diluted 1.1 times. This shows how the dilution value can be decreased by one order of magnitude by increasing the dose rate without actually affecting the metabolic reactions. The free sugar pool in buckwheat (namely glucose, fructose, and sucrose) amounts to about 170 μmoles per gram of dry weight. If the glucose fed equilibrated rapidly with this pool the smallest dose would be diluted about 75 times and the largest 2.3 times. The acetic acid pool in buckwheat was about 14 μmoles per gram of dry weight. This would dilute the smallest dose of acetate about 8 times and the largest dose 1.15 times.

It is apparent that the initial dilution in the endogenous pool can partially account for the pronounced effect of dose rates on the dilution value but other

factors must be considered. For example, the duration of the experiment will probably have a marked effect on the dilution. This has not been investigated very extensively. The data in Table I show that the maximum of a 5-μmole dose of phenylalanine to quercetin was reached after about 8 hours and that 50% of this maximum conversion was reached after only 2 hours. This suggests that the administered phenylalanine was all converted to end products (quercetin, protein, lignins, etc.) by 8 hours' metabolism and that subsequent metabolism only served to dilute these products further. In another experiment (not reported here) using a 5 μmole/g dose, only 15% of the administered activity remained in phenylalanine after 4 hours.

The smaller doses administered in the experiments reported in Table II were probably completely metabolized long before the end of the 24-hour period allowed for metabolism while the larger doses might supply C^{14}-labelled quercetin precursors throughout the experiment. If sufficient experimental time is allowed for all the C^{14} to move out of the intermediate pools into the end products, then the size of these pools will have no effect on the dilution value, which will be determined only by the specific activity of the precursor administered, the percentage of C^{14} converted to the end product, and the amount of preformed and concurrently formed end product. The percentage conversion figures should be constant as long as most of the C^{14} is fixed in the plant but they should fall off if such large doses are given that the pool of precursor is still significantly radioactive at the end of the experiment. This falling off was quite noticeable when large doses of phenylalanine were given, but it is not so evident with glucose and acetate. It may be presumed that the glucose and acetate entered such large, rapidly metabolizing pools that even the largest dose fed was completely metabolized in the experimental time. The free sugar pool was found to be large by analysis and the apparent large size of the acetate pool may be due to rapid equilibration of acetate with intermediates of the citric acid cycle.

The low percentage conversion of small doses of phenylalanine and glucose may have been due to rapid utilization of these substances for other reactions before they were spread throughout the plant sufficiently to partake in quercetin synthesis. In any event there was a definite maximum dose rate for conversion of phenylalanine to quercetin which corresponded to a dose approximately equal in size to the free phenylalanine pool. This maximum is clearly seen if the percentage conversion values are examined but is not immediately obvious if the dilution values are looked at (see Fig. 1).

The purpose of this investigation was to reach a decision on the best way to report results of tracer experiments in which a number of precursors are compared for the biosynthesis of a plant constituent (in this case quercetin).

When comparing C_6–C_3 precursors among themselves if the same dose rate is selected, the inverse ratio of the dilution values is approximately the same as the ratio of the percentage of C^{14} converted. If a C_6–C_3 precursor (B-ring precursor) is compared with another type of precursor (for the A ring or whole molecule) at the same dose rate then the two ways of expressing the results vary, e.g. when acetic acid and L-phenyllactic acids are fed at 7 μmoles per

gram dry weight then according to the dilution values the L-phenyllactic acid is (1300/150) = 8.7 times better than the acetic acid, but using percentage converted values the L-phenyllactic acid is only (3.1/1.4) = 2.2 times better. Similarly in comparing D-glucose and L-phenylalanine fed at 53 and 50 μmoles per gram dry weight respectively the L-phenylalanine is 14.5 times better by dilution values but 5.8 times better by the percentage C^{14} converted.

If C_6–C_3 precursors are compared at the same dose level the dilution values have approximately the same ratio to one another whatever dose is chosen but the selection of any particular dose is arbitrary. If the dose levels vary from precursor to precursor in any one experiment it is difficult to apply a correction to the dilution values to enable one to compare the precursors at the same dose rate.

Precise dilution values are easily obtained in that the only figures needed are the specific activities of two compounds that can be purified to constant specific activity. However, they are very sensitive to variation in the dose at low dose rates. This has been observed for the biosynthesis of phenolic acids in *Salvia splendens* Sello (8) and for the biosynthesis of pungenin in *Picea pungens* (13) although in these experiments only a few results bearing on dose rates were obtained. Dilution values have been used for comparing the efficiency of precursors in lignin biosynthesis (3) because of the difficulties in defining and measuring lignin in plant material.

Precise "percentage converted" figures are more difficult to obtain than dilution figures as they depend on the accuracy of measurement of the amount of product in the plant. In the isolation of rutin by adsorption on Magnesol, recoveries of added rutin were about 90%.

In cases where the amount of product in the plant is so small that, in order to isolate it, excess carrier has to be added, then unless the amount present can be separately measured by some analysis, no dilution value is possible. But from the amount of excess carrier added and its specific activity when finally purified, a value of the total activity in the product (and thus a percentage conversion value) is obtained.

The values for μmoles converted (last column, Table II) take into account the percentage converted and the dose, including any endogenous precursors. They might be preferable to percentage conversion figures for expressing the results of tracer experiments but it is difficult to determine just what the size of the endogenous pool is, particularly since the pool would include any compounds that can equilibrate rapidly with the precursor under the conditions of the experiment. The μmoles converted figures have only been calculated for the larger dose rates and not at all for cinnamic acid and phenyllactic acid where the size of the endogenous pools is unknown. It is noticeable that for acetate, glucose, and phenylalanine the figures for μmoles converted all tend to the approximate figure of 1 to 2 μmoles converted in spite of the large differences in figures for per cent converted. This may represent the actual amount of new quercetin synthesized by the plant under the experimental conditions whichever precursor is administered. For the largest doses of phenylalanine and glucose the μmoles converted are slightly larger and could be explained by a

mass action effect of a pool larger than normal inducing greater quercetin synthesis. In these cases the dose level at which the precursor could still be regarded as without effect on metabolism has been exceeded.

The above arguments apply to end products. If, however, the metabolite under study is an intermediate, a calculation of dilution value can be useful. An intermediate could well contain only a small percentage of the total administered activity, especially if the pathway studied normally plays only a small part in the metabolism of the precursor. However, the dilution value of such an intermediate may be low and if a close relationship exists the specific activity of the intermediate may equal that of the precursor as it exists in the plant at that time. Data of this type are shown in Fig. 1 of McCalla and Neish (8).

Taking everything into consideration it would seem that the percentage conversion value is the most useful way for recording the efficiency of a precursor in a tracer experiment in those cases where reliable analyses of the amount of end product are feasible. It is related to the nearly constant proportion of precursor diverted to product synthesis under tracer conditions and is not so sensitive to variation in dose level as is a dilution value. Also the percentage conversion value eliminates the variation caused by random variation in the amount of preformed product. Actually a better way still would be to express the result as μmole converted per unit time at the initial rate, as is done in enzyme kinetics, but this is too hard to determine experimentally in such a complex system.

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References

1. E. W. Underhill, J. E. Watkin, and A. C. Neish. Can. J. Biochem. and Physiol. **35**, 219 (1957).
2. J. E. Watkin, E. W. Underhill, and A. C. Neish. Can. J. Biochem. and Physiol. **35**, 229 (1957).
3. S. A. Brown and A. C. Neish. Can. J. Biochem. and Physiol. **33**, 948 (1955).
4. S. A. Brown and A. C. Neish. Can. J. Biochem. and Physiol. **34**, 769 (1956).
5. D. Wright, S. A. Brown, and A. C. Neish. Can. J. Biochem. and Physiol. **36**, 1037 (1958).
6. S. A. Brown, D. Wright, and A. C. Neish. Can. J. Biochem. and Physiol. **37**, 25 (1959).
7. D. R. McCalla and A. C. Neish. Can. J. Biochem. and Physiol. **37**, 531 (1959).
8. D. R. McCalla and A. C. Neish. Can. J. Biochem. and Physiol. **37**, 537 (1959).
9. R. J. Emerson and J. T. Van Bruggen. Arch. Biochem. Biophys. **77**, 249 (1958).
10. J. E. Watkin. Chem. & Ind. In press.
11. R. Scarisbrick. Modern methods of plant analysis. Vol. II. Springer-Verlag, Berlin. 1955. p. 444.
12. C. H. W. Hirs, S. Moore, and W. H. Stein. J. Am. Chem. Soc. **76**, 6063 (1954).
13. A. C. Neish. Can. J. Botany, **36**, 649 (1958).

THE PRESENCE OF ANSERINE AND CARNOSINE IN BRAIN TISSUE[1]

E. A. Hosein and Margaret Smart

Abstract

Anserine and carnosine have been found to be present in brain tissue. The dipeptides were isolated by column and paper chromatographic techniques, and their identity was confirmed by subsequent hydrolysis in acid to yield β-alanine, methylhistidine, and histidine.

Introduction

Anserine and carnosine have long been known to be present in muscle tissue (1, 2). In an exhaustive review on the distribution of these dipeptides in nature, du Vigneaud *et al.* (3) found no evidence of their presence in nervous tissue. The metabolic role of these substances in muscle remains obscure, although recently, Severin *et al.* (4) have suggested that anserine, though not carnosine, may be a cofactor in phosphocreatine synthesis. Hosein *et al.* (5) have found that the intramuscular injection of the ethyl ester of gamma-butyrobetaine (gBBE) into rats caused an intense aminoaciduria. Methylhistidine was one of the amino acids excreted. The rat does not normally excrete methylhistidine (6) and accordingly it was believed that in these animals treated with gBBE, this amino acid appeared in the urine as a result of abnormalities of anserine metabolism. In human progressive muscular dystrophy, there is intense aminoaciduria with the excretion of methylhistidine (7). In this disease there is also an increased dipeptidase activity in muscle (8). Chemical analyses of muscle tissue from dystrophics have shown that the muscles are low in phosphocreatine (9). The possible role of anserine in phosphocreatine synthesis has already been mentioned. Hosein and Eisenstein (10) found that intramuscular injections of gBBE into rats produce, among other effects, a low phosphocreatine content of the muscle. From these results it appeared that in dystrophic muscles and muscles of rats treated with gBBE, there could either be increased hydrolysis of anserine or inhibition of its synthesis. In previous experiments Hosein (11) and Hosein *et al.* (12) have shown that during convulsions, betaine esters are released in brain. Other authors have shown that during convulsions the brain phosphocreatine content is low (13). In view of the decreased muscle phosphocreatine observed in rats after treatment with gBBE, it was of interest to determine whether similar effects could be produced in brain during convulsions through the mediation of anserine and carnosine. Accordingly, analysis of brain tissue was first undertaken to isolate these dipeptides. Both anserine and carnosine were found to be present in this tissue.

Materials

Histidine, methylhistidine, β-alanine, and carnosine were obtained from the California Corporation for Biochemical Research.

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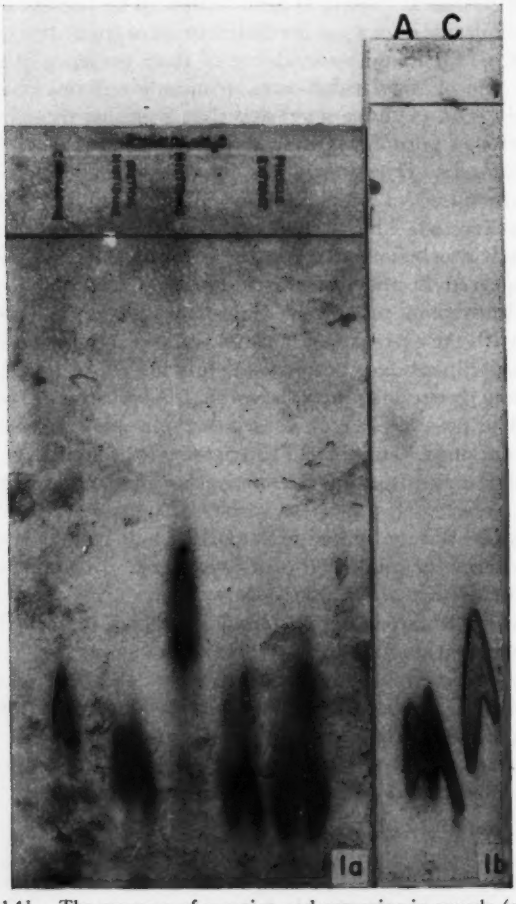
Contribution from the Department of Biochemistry, McGill University, Montreal, Que.

The Sprague–Dawley rats used in these experiments were normal adults of either sex but of an average weight of 150 g.

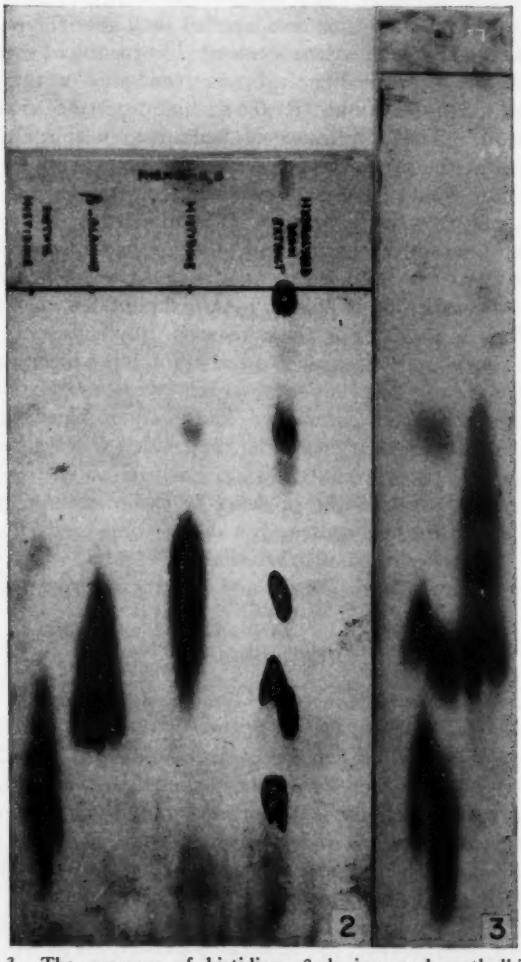
Experimental and Results

Anserine and carnosine were extracted from normal rat brain and muscle tissue by the method of McManus (14). Twenty grams of fresh rat brain or muscle was deproteinized by homogenizing with a 10-fold volume of 1% picric acid followed by centrifugation. The supernatant solution was freed from picric acid by passage through a column (2×8 cm) containing Dowex 2-C1, as



FIGS. 1*a* and 1*b*. The presence of anserine and carnosine in muscle (*a*) and brain (*b*) extracts. In Fig. 1*b* is shown the chromatogram of the individual dipeptides anserine (A) and carnosine (C) from brain tissue. These substances were obtained on eluting individual sections of the chromatogram for the brain extract which corresponded to those of the dipeptides shown in Fig. 1*a*. The concentrated eluates were then rechromatographed in the phenol–water system.

recommended by McManus. The effluent was concentrated to 10 ml under reduced pressure at 40° C and aliquots were passed through a smaller column (0.8×15 cm) containing Dowex 50X 4 in the hydrogen form, to adsorb the dipeptides. The 0.1 *M* pyridine eluate containing the dipeptides was reduced to dryness at 40° C and 0.3 ml distilled water was added. One-tenth milliliter of this solution was applied as a spot for paper chromatographic analysis in the phenol–water (3–1) system. The developed chromatogram was sprayed with a 1% solution of ninhydrin in ethanol. The results of typical experiments are shown in Fig. 1*a*. Two spots were found on the chromatogram made from muscle extract, one of which corresponded to carnosine (R_f 0.72) and the other



FIGS. 2 and 3. The presence of histidine, β-alanine, and methylhistidine in the hydrolyzate of dipeptides obtained from brain tissue extracts.

was assumed to be anserine (R_f 0.85), since no authentic material was available. These results are in good agreement with those of McManus (14), who also used muscle tissue and authentic anserine. The results obtained with brain extracts, Fig. 1*b*, were very similar to those obtained with muscle extracts.

In order to confirm the presence of anserine and carnosine, McManus (14) has suggested acid hydrolysis of the dipeptides with subsequent chromatographic analysis of the products. Accordingly, a portion, 0.2 ml of the concentrated eluate made earlier, was transferred to a Pyrex test tube, 5 ml of 6 *N* HCl was added, and the tube was sealed. The sealed specimen was heated in an oil bath for 5 hours at 110° C. The tube was cooled, broken, and the contents were evaporated to dryness. The residue was dissolved in 0.5 ml distilled water, and 0.1 ml of this solution was applied as a spot for paper chromatographic analysis in the phenol–water system. The results of these experiments with brain tissue are shown in Fig. 2. Spots were found on the chromatogram which corresponded to β-alanine (R_f 0.63), histidine (R_f 0.55), and methylhistidine (R_f 0.80). The appearance of β-alanine on this chromatogram is further evidence for the presence of anserine and (or) carnosine in the tissues, since free β-alanine would have been removed from the extract earlier during the column chromatographic separation. It is difficult to separate and identify mixtures of histidine and carnosine and anserine and methylhistidine in the phenol–water system. In such mixtures, the presence of β-alanine after acid hydrolysis is the best method of identifying the dipeptides when paper chromatographic analysis is used. For these reasons, duplicate chromatograms of those shown in Fig. 1*a* and 1*b* for muscle and brain, but which were not sprayed with ninhydrin, were carefully cut so as to remove separately the spots which corresponded to anserine and carnosine. These bits of paper were eluted with water, the eluate reduced to dryness, and the residue was treated with HCl as described above. Paper chromatographic analysis of each solution in the phenol–water system revealed the presence of β-alanine and histidine in the hydrolyzate of the carnosine spot and β-alanine and methylhistidine in the hydrolyzate of the anserine spot. From these results, which are shown in Fig. 3, it appears that in both muscle and brain, carnosine and anserine are present.

Discussion

Relatively little is known of the metabolic role and function of the dipeptides anserine and carnosine. Even less is known of the roles of the amino acids β-alanine and methylhistidine. However, Bazemore *et al.* (15) have observed that β-alanine inhibits the stretch receptor of the crayfish and Hosein *et al.* (16) have found that methylhistidine is a neuromuscular blocking agent in the cat. The concentration of β-alanine in brain is very small (17) and from data obtained in unpublished experiments, it appears that the methylhistidine concentration could be of the same order. Recently, Severin *et al.* (4) have presented evidence which suggests that anserine, but not carnosine, could be a cofactor in the phosphorylation of creatine. Experiments by Hosein *et al.* (10) have shown that the intramuscular injection of gBBE into rats caused a

decreased muscle phosphocreatine and ATP content. Other studies in our laboratory (unpublished) have shown that gBBE caused a stimulation of muscle ATP-ase activity in vitro. Accordingly, the decreased phosphocreatine content found in the muscles of animals treated with gBBE could be due to the diminished ATP content, inhibition of creatine kinase, or inhibition of anserine synthesis. Previous experiments (4) have shown that rats treated with gBBE excrete methylhistidine in the urine. On the basis of results presented by Winnick *et al.* (18) and Kalyankar *et al.* (19), the methylhistidine excreted could have originated from the unused amino acid consequent to inhibition of synthesis of the dipeptide, but it might also be produced through the excessive hydrolysis of the anserine by anserinase under our experimental conditions. Earlier studies by Hosein (11) have shown that the intracranial injection of 100 μg gBBE into unanaesthetized rats caused violent convulsions which were fatal. Since it has been reported that during convulsions the phosphocreatine content of brain is low, the influence of gBBE on the metabolism and function of anserine and carnosine in brain tissue is currently being investigated.

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References

1. W. Gulewitsch and S. Admiradzibi. Ber. **33**, 1902 (1900).
2. D. Ackermann, O. Timpe, and K. Poller. Z. physiol. Chem. **183**, 1 (1929).
3. V. du Vigneaud and O. K. Behrens. Ergeb. Physiol. **41**, 917 (1939).
4. S. E. Severin, N. P. Meshkova, and N. I. Rasumovskaya. Doklady Akad. Nauk. S.S.S.R. **103**, 871 (1955).
5. E. A. Hosein, M. Smart, and K. Hawkins. In preparation.
6. S. P. Datta and H. Harris. Nature, **168**, 296 (1951).
7. S. R. Ames and H. A. Risley. Proc. Soc. Exptl. Biol. Med. **68**, 131 (1948).
8. I. M. Weinstock, A. D. Goldrich, and A. T. Milhorat. Proc. Soc. Exptl. Biol. Med. **91**, 302 (1957).
9. J. G. Reinhold and G. R. Kingsley. J. Clin. Invest. **17**, 377 (1938).
10. E. A. Hosein and S. Eisenstein. In preparation.
11. E. A. Hosein. To be published.
12. E. A. Hosein and P. Proulx. Nature. In press.
13. W. E. Stone, J. E. Webster, and E. S. Gurdjian. J. Neurophysiol. **8**, 233 (1945).
14. I. McManus. J. Biol. Chem. **225**, 325 (1957).
15. A. Bazemore, K. A. C. Elliott, and E. Florey. Nature, **178**, 1052 (1956).
16. E. A. Hosein, E. Ottolenghi, and S. Dorfman. 2nd International Symposium on Myasthenia Gravis. H. R. Viets (*Editor*). Charles C. Thomas, Springfield. In press.
17. C. E. Dent. Biochem. J. **43**, 168 (1948).
18. R. E. Winnick and T. Winnick. Biochim. et Biophys. Acta, **31**, 47 (1959).
19. G. D. Kalyankar and A. Meister. J. Biol. Chem. **234**, 2310 (1959).

EFFECTS OF INFLAMMATION ON PROTEINS AND ENZYMES IN RAT PLASMA[1]

S. W. Levy and de Guise Vaillancourt

Abstract

The effects of anaphylactoid inflammation in rats on the limb volume, hematocrit, and plasma proteins, and on the activities of plasmin, antiplasmin, and esterase of the plasma were studied. The hyperemia and edema of the limbs and other extremities which occurred following the injection of dextran were found to be accompanied by a transient hemoconcentration and a prolonged depression in the concentration of plasma proteins (both albumin and globulin fractions). The specific activity of plasma esterase was increased and that of antiplasmin was decreased during inflammation compared with control animals. No consistent or significant change was observed in the specific activity of plasmin.

Introduction

Anaphylactoid inflammation is the characteristic response of rats to a single injection of egg white (1–3), dextran (4–8), and other materials (6–10). The reaction consists of an intense, transient hyperemia and swelling of the limbs and extremities which develops shortly after the injection and subsides several hours later. This phenomenon has been shown to be associated with the "explosive" destruction of the mast cells (7, 8, 11) which liberate their stores of histamine (3, 8, 12–14) and 5-hydroxytryptamine (8, 15, 16). Histamine liberators such as compound 48/80 (8, 15, 16) and 1935L (16) also induce the reaction while antihistamines and particularly inhibitors of 5-hydroxytryptamine (dibenamine, chlorpromazine, and promethazine (phenergan)) are known to prevent or attenuate it (2, 5, 8, 16, 17). It has also been shown that repeated injections of egg white (1, 6), dextran (4, 6, 14), or compound 48/80 (7, 9, 18) ultimately destroy the ability of rats to respond, possibly owing to the exhaustion of the mast cell stores (7–9, 18).

Although histamine, following its liberation from mast cells or other sources, is known to cause vasodilation and an increase in permeability of the capillaries, the mechanisms of inflammation and edema in anaphylactoid or immunologic reactions and in human diseases of the joints and connective tissue are not fully understood. The possibility that certain hydrolytic enzymes may participate in the inflammatory process either by disrupting the mast cells or by nonspecific damage to the capillary and other membranes led us to investigate the activities of plasmin (fibrinolysin), antiplasmin, and esterase in the plasma of rats subjected to anaphylactoid inflammation and edema.

Methods

Female Wistar strain rats weighing 150–250 g were anaesthetized with ether, the jugular vein was exposed, and a blood sample (0.6 ml) was drawn with a syringe containing 1/10 volume of 3.8% sodium citrate solution. The incision

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was closed with Michel clamps and the rats were injected intraperitoneally with 1 ml of either 6% glucose in saline as controls, or with Abbott's 6% dextran in saline, to induce inflammation. After various periods of time the rats were anaesthetized again, limb volume measurements were made (10), the incision was reopened, and a further blood sample was drawn; glucose (6%; 0.6 ml) was injected intraperitoneally after each sample. After it was mixed immediately with the citrate solution, the blood sample was deposited directly into a hematocrit tube, chilled, and centrifuged at 3000 r.p.m. for 15 minutes. The hematocrit was recorded and the plasma removed.

Rat plasmin was assayed by activating the enzyme (see below) and measuring its digestive power toward casein, as previously described (19).

Following the precipitation of plasma euglobulins by means of dilution at low pH (20), antiplasmin was estimated by performing plasmin assays on normal rat plasma euglobulin in the presence and absence of portions of the supernatant albumin fractions obtained from the plasma of experimental rats. A summary of the method used for the determination of rat antiplasmin is represented in the following series of reactions.

1. Human globulin, "proactivator" (8 volumes) + streptokinase (1.0%; 3 volumes) $\xrightarrow[37° C]{10 \text{ min}}$ "activator"

2. Rat plasminogen, euglobulin + "activator" (5.0%; v/v) $\xrightarrow[37° C]{2 \text{ hr } 40 \text{ min}}$ rat plasmin

3. Rat plasmin (4 volumes) (pooled, standard)
   - + antiplasmin supernat. (3 volumes) $\xrightarrow[37° C]{20 \text{ min}}$ plasmin–antiplasmin
   - + 0.016% acetic acid (3 volumes) $\xrightarrow[37° C]{20 \text{ min}}$ control plasmin

The proportion of antiplasmin supernatant routinely employed, i.e. 75% v/v, was chosen on the basis of a preliminary experiment which showed that normal antiplasmin caused 50% inhibition of the standard plasmin at the above concentration. The results were expressed as the percentage inhibition of standard plasmin solutions under the conditions of these assays.

Tributyrin esterase was determined manometrically in the Warburg apparatus (21). Tributyrin (0.03 ml) and water (2.4 ml) were placed in Warburg flasks and sodium bicarbonate solution (1.2%; 0.5 ml) and rat plasma (0.06 ml) were added to the side-arms. The vessels were gassed for 15 minutes with 5% CO_2 in N_2, equilibrated at 37° C, and the contents mixed. Readings were taken at 10-minute intervals for a period of 40 minutes, the initial reading being discarded in estimating the rate of evolution of CO_2. Plasma esterase activity was expressed in μl CO_2 evolved /0.06 ml /30 minutes.

Protein nitrogen in the whole plasma and albumin–"pseudoglobulin" fraction was determined by the method of Lowry *et al.* (22) using the Folin–Ciocalteu phenol reagent. The quantity of euglobulin was calculated by difference. A solution of crystalline bovine serum albumin (Armour) containing 10.0 mg N per 100 ml was used as the standard.

The observation that variations occurred in the quantity of euglobulin precipitated from the plasma of experimental rats led us to determine routinely

the optical density at 650 mμ after dilution of the plasma samples with 19 volumes of 0.016% acetic acid.

Results

Effects of in vitro Dextran

As a control study on the effects of dextran-induced inflammation in rats, an experiment was performed to determine the possible influence of dextran in vitro on the plasmin, antiplasmin, esterase, and euglobulin optical density of normal rat plasma. Dextran was added in various concentrations to samples of normal rat plasma which were then distributed and assayed for the above-mentioned activities. The results of this experiment together with a statistical summary of the data are shown in Table I. Since the variations observed within these groups of data lie within the respective ranges of experimental error, it is evident that the activity of plasmin, antiplasmin, and esterase and the optical density of the euglobulins were not affected at concentrations of dextran up to five times the possible maximum in the plasma of experimental rats.

TABLE I

Effects of dextran in vitro on plasmin, antiplasmin, tributyrin esterase, and euglobulins of rat plasma

Added dextran (g%)	Plasmin (O.D. inc./0.2 ml/hr)	Antiplasmin (% inhibition)	Tribut. esterase (μl CO_2/0.06 ml/30 min)	Euglobulin O.D. at 650 mμ
0	154	52	106	220
0.23*	155	54	124	213
0.46	149	59	124	208
0.69	160	57	121	200
1.16	154	52	112	202
Mean ± S.E.	154±2	55±1.5	117±4.0	209±4.0
C =	2.6%	5.8%	6.9%	3.9%

*Estimated maximum concentration of dextran in plasma of rats subjected to anaphylactoid inflammation.

Effects of in vivo Dextran

The results to be described are those of a typical experiment performed on three rats, one being a control animal which had been injected with glucose, the other two having received dextran intraperitoneally to induce inflammation. Although the dextran-injected rats were treated identically in all respects, the results obtained for both animals are included because they serve to illustrate some of the variability encountered in the series of experiments which they represent, i.e. 10 glucose controls, 20 dextran-treated rats. In order to illustrate the changes relative to the control animal, the data have been plotted as percentages of the control values obtained prior to the injections.

Changes in Limb Volume and Hematocrit

Figure 1*a* indicates the characteristic development of edema in the limbs following intraperitoneally administered dextran. The increase in limb volume was always accompanied by intense reddening of the limbs and mucous membranes, which seemed to precede the onset of the edema and to subside sooner. The limb volume generally attained a maximum about one hour after the injection but in some experiments this occurred in 2 hours or longer. It should be noted here that a few rats responded to the dextran with obvious

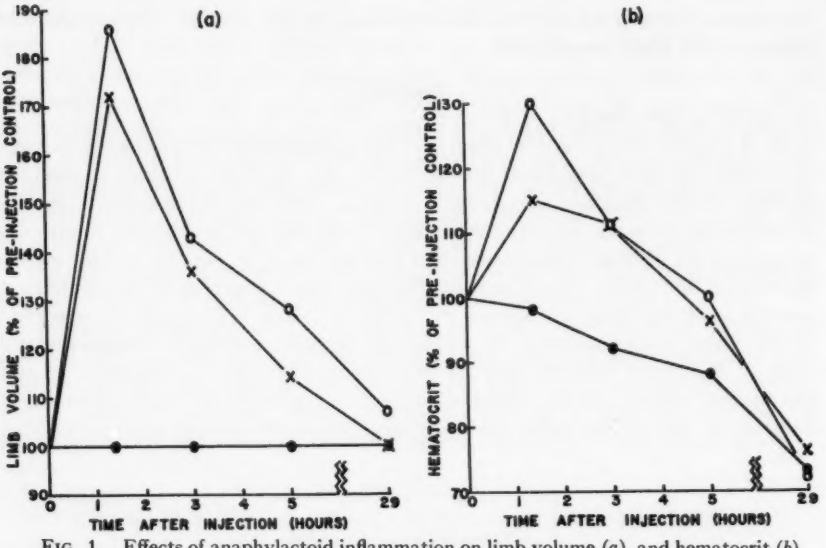

FIG. 1. Effects of anaphylactoid inflammation on limb volume (*a*), and hematocrit (*b*) of rats. ●——●, rat No. 1, glucose-injected control; ○——○, rat No. 2, dextran (6%; 1.0 cc) injected intraperitoneally at zero time; ×——×, rat No. 3, dextran-injected duplicate. Data are expressed as percentages of the zero time, preinjection control values.

reddening of the extremities and mucous membranes but without evidence of edema. This response may have been due either to the inherent resistance of certain rats to the anaphylactoid reaction or to some external factor such as too recent feeding of the animals (in earlier experiments) or the use of a depolymerized dextran solution. No significant change has ever been observed in the appearance or volume of the limbs in the glucose-injected control rats.

The changes in hematocrit shown in Fig. 1*b* are typical of those observed in all the experiments, i.e., a steady decline in values for the control animal and an initial rise and subsequent fall for the experimental rats. The fall in hematocrit which represents progressive hemodilution may be accounted for by the replacement of withdrawn blood by tissue fluid and injected glucose. In dextran-treated rats, the initial hemoconcentration evidently reflects the escape of plasma fluid into the tissues and the development of edema, as described in Fig. 1*a*.

Changes in Plasma Protein Concentration

Figure 2 indicates the changes in protein concentration of the plasma and plasma fractions in the control and dextran-treated rats. For total plasma proteins and the albumin–pseudoglobulin fraction (Figs. 2*a*, 2*b*), it is evident that the two sets of curves are remarkably similar. The control animal underwent no significant change while the dextran-treated rats suffered a 30% reduction in protein concentration during the inflammatory period and their condition returned to normal by the following day. The curves for euglobulin concentration (Figs. 2*c*, 2*d*) indicate essentially the same effects with respect

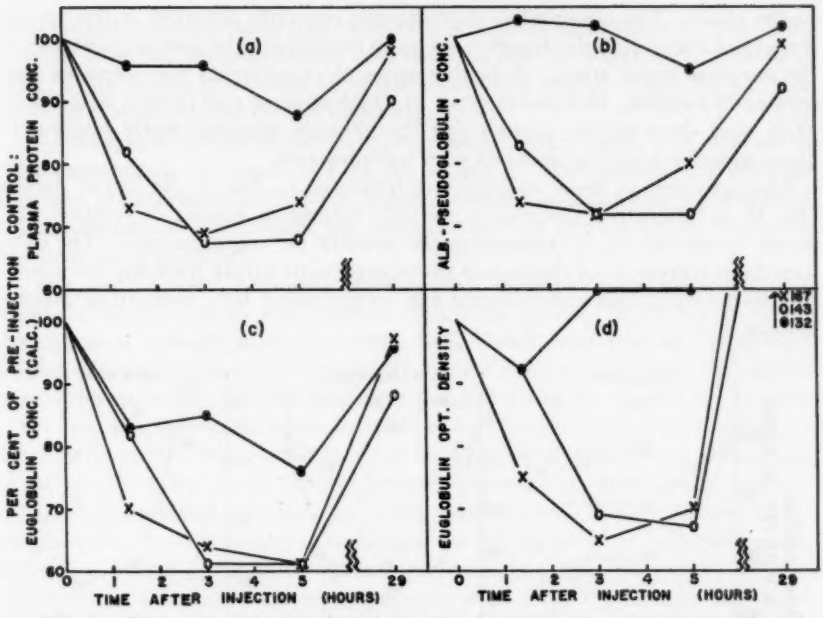

FIG. 2. Effects of anaphylactoid inflammation in rats on the concentration of total plasma protein (*a*), albumin–pseudoglobulin (*b*), and euglobulin ((*c*), calculated; (*d*), optical density at 650 mμ (see text)). ●——●, rat No. 1, glucose-injected control; ○——○, rat No. 2, dextran (6%; 1.0 cc) injected intraperitoneally at zero time; ×——×, rat No. 3, dextran-injected duplicate. Data are expressed as percentages of the zero time, preinjection control values.

to the difference between the control and dextran-treated rats. However, in the measurement of euglobulin optical densities (Fig. 2*c*), the 29-hour samples yielded much higher values than the preinjection control samples as observed in many experiments, and in Fig. 2*d* the euglobulin concentration for the control animal, calculated by subtracting the value for the pseudoglobulin from that of the total plasma protein, showed a fall in values during the early experimental period.

In view of the hemoconcentration which occurred in dextran-treated rats (Fig. 1*b*), reflecting the escape of fluid from the circulation, the reduction in plasma protein concentration is not readily explained. It may be noted that whereas the limb volume and hematocrit were returning rapidly to normal by the third hour, the concentration of proteins fell to a minimum at this time and remained depressed at the fifth hour after the injection. This suggests that a process was in operation which was independent of the migration of protein and water between blood and tissues during inflammation.

Changes in Plasmin, Antiplasmin, and Tributyrin Esterase

The effects of inflammation and edema on plasmin, antiplasmin, and esterase activities of the plasma in rats were initially explored (5 controls, 10 experimental rats) without reference to the possible changes in protein concentration

of the blood. The observation that changes regularly occurred in the optical density of the euglobulin fraction led us to investigate the specific activities of the enzymes under study. A further series of experiments was therefore performed (5 controls, 10 experimental) in which enzyme and protein determinations were done on the plasma and the albumin–pseudoglobulin fraction of blood samples from rats induced with inflammation.

The activities of these enzymes are indicated in Fig. 3, the upper curves (*3a*, *3b*, *3c*) depicting the results per unit volume of plasma or fraction, the lower curves (*3d*, *3e*, *3f*) expressing the activity per mg of protein. The data have been corrected for dilution of the plasma with citrate with due consideration to the variations in hematocrit and an estimated 10% interstitial plasma volume.

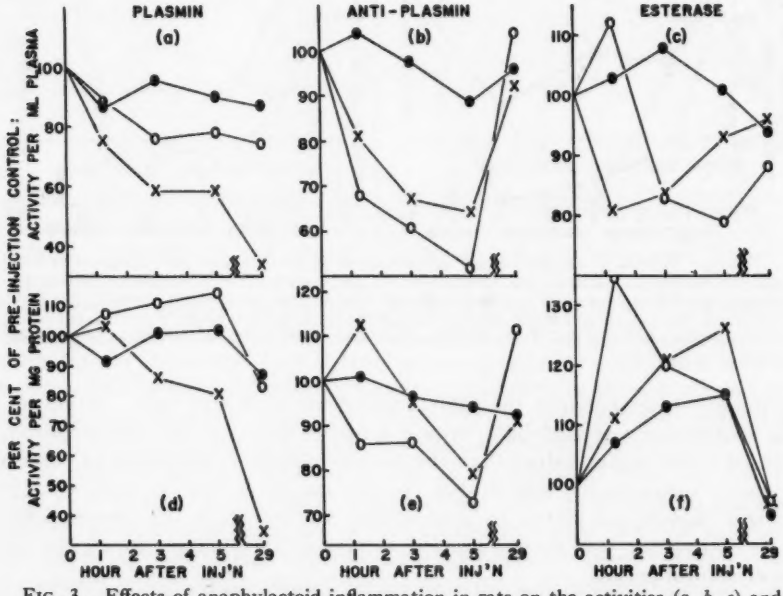

FIG. 3. Effects of anaphylactoid inflammation in rats on the activities (*a*, *b*, *c*) and specific activities (*d*, *e*, *f*) of plasmin, antiplasmin, and tributyrin esterase respectively. The specific activities of plasmin and esterase are based upon total plasma protein concentrations, those of antiplasmin, upon the concentrations of albumin–pseudoglobulin. Data are expressed as percentages of the zero time, preinjection control values. ●——●, rat No. 1, glucose-injected control; ○——○, rat No. 2, dextran (6%; 1.0 cc) injected intraperitoneally at zero time; ×——×, rat No. 3, dextran-injected duplicate.

Figures *3a*, *3b*, and *3c* show that the activity of plasmin, antiplasmin, and esterase was depressed during anaphylactoid inflammation in rats. Similar results were obtained in many other experiments. In the case of plasmin, however, as seen in Fig. *3a*, the differences between the results of the control and experimental rats were sometimes in the order of 10% or less and therefore not significant. The profound fall in activity which occurred in rat No. 3 on the following day (29 hours after dextran) has been observed occasionally in

other experiments and sometimes occurred 4–5 hours after the injection. The activity of both the antiplasmin and esterase fell by about 40% and 20% respectively during the course of inflammation and edema (Figs. 3*b*, 3*c*), and returned toward normal by the following day.

The changes in specific activities of the plasmin, antiplasmin, and esterase in these experiments are shown in Figs. 3*d*, 3*e*, and 3*f*. The plasmin and esterase activities are expressed in terms of plasma protein concentrations while that of the antiplasmin is based upon the concentrations of protein in the albumin–pseudoglobulin fractions.

The specific activity curves for plasmin (Fig. 3*d*) were typical of the variable results obtained in many experiments. While the activity of rat No. 2 during the course of inflammation was about 10% higher than that of the control animal, rat No. 3 apparently responded by a fall in activity to about 20% below that of the control by the fifth hour after the administration of dextran. In view of the fact that these data are based upon two distinct sets of determination, we consider that little or no significance can be attached to the differences observed in the specific activities of plasmin between control and experimental rats.

Figure 3*e* indicates the influence of inflammation on the specific activity of antiplasmin. Similar results have been observed consistently in other experiments, i.e. a general tendency toward lower values of the order of 20–25% in rats induced with inflammation, compared with control animals. Since the maximum difference tended to occur toward the fifth hour in this and other experiments, rather than during the active phase of inflammation (cf. Fig. 1*a*), the reduction in antiplasmin would appear to be a result rather than a cause of the inflammatory process.

The changes in specific activity of plasma esterase are indicated in Fig. 3*f*. It is evident that the activity in the experimental animals was increased compared to that of the control. This differential effect, though small, has been observed in most of the experiments and like the changes in antiplasmin, tended generally to occur toward the fifth hour after the administration of dextran. The unusual rise in activity in rat No. 2 in the first hour has not been observed in other experiments. In another experiment the esterase declined to a value of 40% of the control after 6 hours and remained low on the following day.

Discussion

The present studies have shown that the injection of dextran intraperitoneally into rats gives rise to a group of physiological reactions which include the following:

(*a*) intense hyperemia and reddening of the limbs, ears, and mucous membrane regions (lips, tongue, nasal openings, clitoris) which occurs in 10–15 minutes and persists for 3 hours following the injection; this reaction is accompanied by a transient exophthalmos, previously noted in histamine-treated rats (23);

(*b*) swelling of the limbs, face, snout, and perineal region, commencing in 15–20 minutes and persisting for 6–8 hours;

(*c*) a rise in hematocrit during the first hour, which corresponds to the initial period of rapid swelling;

(*d*) a reduction of about 30% in plasma protein concentration (both albumins and globulins) within 3 hours after the injection. The hypoproteinemia persists for 6 hours and returns to normal by the following day;

(*e*) a small, but fairly consistent increase in the specific activity of plasma tributyrin esterase;

(*f*) a consistent reduction in the specific activity of antiplasmin to a minimum of ca. 20% below the control value at the fifth hour;

(*g*) no consistent or significant change in the specific activity of plasmin.

The development of edema, in our experience, was invariably preceded by the intense hyperemia, which also subsided more quickly. Also, as mentioned previously, some rats which failed to develop a measurable edema after dextran nevertheless displayed reddening of the limbs, ears, and mucous membrane regions. Since both reactions occur in the same body areas, it would appear that either vasodilation is a necessary stage in the development of edema or that these are distinct and independent phenomena. Since the effects of dextran in rats are mediated by the liberation of histamine (8, 12–14, 16) and 5-hydroxytryptamine (8, 16), either of which exerts independent actions in dilating and increasing the permeability of the capillaries (2, 8, 16), these reactions are evidently not related. The role of the "slow-reacting substance" (25–27) which also is released during anaphylactoid inflammation in rats (14) has not been defined.

The transient increase in the hematocrit which occurs within the first hour after the intraperitoneal injection of dextran is readily explained by the exudation of fluid from the circulation and the resulting edema of the tissues. Similar results have been observed during egg-white edema in rats (3). Both the rise in hematocrit and its return to control-rat values were consistently found to coincide in time with the changes in limb volume. Halpern and Briot (5, 28) have observed that the antihistamine phenergan or small doses of adrenalin, which prevent inflammation, also prevent hemoconcentration. These observations, together with the results of experiments showing the migration of injected dye into the tissues (8, 29, 30), were considered as evidence that the primary lesion of anaphylactoid inflammation is an increase in permeability of the capillaries. The potentiation of the anaphylactoid reaction by insulin (10) and the absence of the reaction in experimental diabetes (31, 32) also may be related to the action of insulin in increasing membrane permeability (33), thus inducing a state of hypersensitivity of the animal by permitting a freer exchange of materials between the blood and tissues (31).

Several possible mechanisms may account for the reduction in plasma protein concentration during inflammation. The simplest explanation is that plasma proteins escape or are transported (34) into the tissues where they are trapped and replaced in the circulation by water and intracellular potassium (35) from the tissues. Although this mechanism would account for the prolonged depression of plasma proteins relative to the period of edema formation

(Figs. 1*a*, 2), it does not explain the rapid initial fall in plasma protein concentration, which implies that protein is being expelled into the tissues in a higher concentration than that which occurs in plasma. It should be noted here that the protein content of edema fluid found in experimental rats is less than half that of plasma (29). In seeking an alternative explanation for this effect, two other possible mechanisms were considered.

(1) A transient functional impairment of the kidneys, permitting the rapid loss of proteins. In this regard, we have found no reference in the literature to suggest that this may occur; nor has the kidney been noted as a mast cell-containing (36) shock organ (7, 37) in the rat.

(2) The activation or liberation of a protease, resulting in enzymatic degradation of the plasma proteins. Proteolytic activity induced by anaphylactoid or antigen–antibody reactions (38, 39) could conceivably act directly upon the mast cell proteins to liberate histamine, 5-hydroxytryptamine, and other substances, or could release vasodilator and inflammatory polypeptides (27, 40) from the plasma proteins.

Although our studies have revealed no increase in plasma protease activity during anaphylactoid inflammation, the following points should be considered.

(*a*) Our method for estimating plasmin activity in rat plasma (19) involves the use of a nonphysiological activating system and therefore measures the concentration and potential activity of plasminogen rather than the plasmin activity which may be associated with the inflammatory process.*

(*b*) The reduction in specific activity of antiplasmin seen in our experiments indicates that plasmin may be freed, to some extent, of normal inhibition during inflammation.

(*c*) The participation of proteolysis in anaphylactoid and immunologic reactions has been indicated by the work of many investigators (12, 26, 38, 39, 41, 42), although its exact role is not yet clearly defined.

The increase in specific activity of plasma esterase during inflammation serves to remind us that lipolytic as well as proteolytic enzymes and their products are known to cause histamine release (cf. 11). However, our studies in no way show that the nonspecific esterase of rat plasma functions in this manner. The slight increase in plasma esterase observed in most experiments may have been secondary to the inflammation reaction, resulting either from the passage of tissue esterases into the blood or possibly from the presence of an esterase induced by heparin (43–45) following its liberation from the mast cells (cf. 46, 47).

Acknowledgments

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References

1. H. Selye. Endocrinology, **21**, 169 (1937).
2. J. Léger, G. Masson, and J. L. Prado. Rev. can. biol. **6**, 359 (1947).

*In recent experiments, we have been unable to detect the presence of spontaneous, non-activated caseinolytic activity in the plasma euglobin of experimental rats.

3. M. SCHACHTER and J. TALESNIK. J. Physiol. **118**, 258 (1952).
4. A. B. VOORHEES, J. BAKER, and E. J. PULASKI. Proc. Soc. Exptl. Biol. Med. **76**, 254 (1950).
5. M. BRIOT and B. N. HALPERN. Compt. rend. soc. biol. **146**, 15, 21 (1952).
6. R. HOENE, P. LABBÉ, and H. SELYE, Proc. Soc. Exptl. Biol. Med. **79**, 65 (1952).
7. G. JASMIN. Rev. can. biol. **15**, 107 (1956).
8. D. A. ROWLEY and E. P. BENDITT. J. Exptl. Med. **103**, 399 (1956).
9. W. FELDBERG and J. TALESNIK. J. Physiol. **120**, 550 (1953).
10. V. W. ADAMKIEWICZ, W. B. RICE, and J. D. MCCOLL. Can. J. Biochem. and Physiol. **33**, 332 (1955).
11. W. FELDBERG. J. Pharm. and Pharmacol. **6**, 281 (1954).
12. B. N. HALPERN and M. BRIOT. Compt. rend. soc. biol. **146**, 1552 (1952).
13. B. N. HALPERN and M. BRIOT. Compt. rend. soc. biol. **148**, 959 (1954).
14. B. N. HALPERN and M. BRIOT. Compt. rend. soc. biol. **148**, 1046 (1954).
15. B. K. BHATTACHARYA and G. P. LEWIS. Brit. J. Pharmacol. **11**, 202 (1956).
16. B. N. HALPERN, P. LIACOPOULOS, and M. LIACOPOULOS-BRIOT. Compt. rend. soc. biol. **151**, 1692 (1957).
17. M. KRAMER. Arch. exptl. Pathol. Pharmakol. **228**, 302 (1956).
18. V. W. ADAMKIEWICZ and Y. L. LANGLOIS. Can. J. Biochem. and Physiol. **36**, 483 (1958).
19. S. W. LEVY. Can. J. Biochem. and Physiol. **37**, 245 (1959).
20. E. E. CLIFFTON and D. A. CANNAMELA. Blood, **8**, 554 (1953).
21. P. RONA and A. LASNITZKI. Biochem. Z. **152**, 504 (1924).
22. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. J. Biol. Chem. **193**, 265 (1951).
23. W. T. LONGCOPE. J. Exptl. Med. **36**, 627 (1922).
24. F. C. MACINTOSH. Ann. Otol. Rhinol. & Laryngol. **65**, 385 (1956).
25. W. D. M. PATON. Brit. J. Pharmacol. **6**, 499 (1951).
26. W. T. BERALDO. Am. J. Physiol. **163**, 283 (1950).
27. G. P. LEWIS. J. Physiol. **140**, 285 (1958).
28. B. N. HALPERN and M. BRIOT. Arch. intern. pharmacodynamie. **91**, 291 (1952).
29. B. N. HALPERN. La Presse Med. **57**, 949 (1949).
30. A. A. MILES and E. M. MILES. J. Physiol. **118**, 228 (1952).
31. A. GOTH, W. L. NASH, M. NAGLER, and J. HOLMAN. Am. J. Physiol. **191**, 25 (1957).
32. V. W. ADAMKIEWICZ and L. M. ADAMKIEWICZ. Am. J. Physiol. **197**, 377 (1959).
33. E. J. ROSS. Medicine, **35**, 355 (1956).
34. J. F. ALKSNE. Quart. J. Exptl. Physiol. **44**, 51 (1959).
35. B. N. HALPERN, M. BRIOT, D. MOUTON, and J. TRUFFERT. Compt. rend. soc. biol. **149**, 1223 (1955).
36. J. F. RILEY and G. B. WEST. J. Physiol. **120**, 528 (1953).
37. H. SELYE. J. Allergy, **25**, 97 (1954).
38. G. UNGAR. Intern. Arch. Allergy Appl. Immunol. **4**, 258 (1953).
39. G. UNGAR and E. DAMGAARD. J. Exptl. Med. **101**, 1 (1955).
40. D. L. WILHELM, A. A. MILES, and M. E. MACKAY. Brit. J. Exptl. Pathol. **36**, 8 (1955).
41. E. E. CLIFFTON. J. Lab. Clin. Med. **39**, 105 (1952).
42. W. B. GEIGER. J. Immunol. **68**, 11 (1952).
43. S. W. LEVY and R. L. SWANK. J. Physiol. **123**, 301 (1954).
44. S. W. LEVY and R. L. SWANK. J. Physiol. **127**, 297 (1955).
45. D. K. MYERS, A. SCHOTTE, and B. MENDEL. Biochem. J. **60**, 481 (1955).
46. J. F. RILEY. Pharmacol. Revs. **7**, 267 (1955).
47. S. W. LEVY. Rev. can. biol. **17**, 1 (1958).

EFFECT OF MAGNESIUM DEFICIENCY ON THE REGENERATION OF MAST CELLS AFTER TREATMENT WITH 48/80 IN RATS

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Abstract

The decrease in subcutaneous mast cell population observed in rats on a magnesium-deficient diet has been confirmed. Pretreatment with histamine liberator compound 48/80 accentuates the decrease in mast cell counts. This condition was apparently the result of defective regeneration. The decrease in the number of subcutaneous mast cells was accompanied by a parallel reduction of histamine as appreciated by the guinea pig ileum assay. The peripheral vasodilation, which is an early sign of magnesium deficiency, did not appear in rats pretreated with 48/80.

Introduction

Recently Bélanger *et al.* (1) reported that rats fed a diet deficient in magnesium showed a reduction of dermal mast cells, as well as the peripheral vasodilation characteristic of magnesium deficiency (2). This observation suggests that magnesium is essential for the maintenance of the mast cell. In the present study, a histamine liberator, 48/80 (condensation product of paramethoxyphenethylmethylamine and formaldehyde) was administered to rats before they were placed on a magnesium-deficient diet. This agent is known to induce a decrease in the population of mast cells as well as in the content of histamine (3, 4) and of serotonin (5, 6) in the tissues of the rat. The object was to determine to what extent mast cells can regenerate on a magnesium-deficient diet and whether or not the presence of mast cells is necessary for the hyperemic reaction to appear.

Experimental Procedures

Six groups of 80-g (range 72–90) male rats were used, as shown in Table I. Magnesium deficiency was produced by feeding a casein diet containing less than 2 p.p.m. of magnesium, prepared by General Biochemicals. Groups I and III, used as controls, received the diet into which had been blended 0.6 g of magnesium sulphate per 100 g of diet. Groups II, IV, and VI, which also received the control diet, were treated for 12 days with increasing doses of 48/80, starting with 50 μg administered subcutaneously twice daily with regular increases to 500 μg on the 10th day; this last dose was then injected intraperitoneally once daily for the last 2 days. Group II was killed immediately after the 12 days of treatment with 48/80, together with control Group I; Group IV was kept on the control diet whereas Group VI as well as Group V were given the magnesium-deficient diet; these groups (Groups III to VI) were killed 40 days later.

Autopsy was made after fixation by perfusion with isotonic formalin. Portions of skin from the face and abdomen were embedded in paraffin and

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Contribution from the Department of Histology and Embryology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario.

cut at 10 μ; every 10th section was stained with diluted Wright's stain for mast cell counts (1). Four to six sections were cut in each case. Counts were made at a magnification of 356, which produces a microscopic field of 0.335-mm diameter; cells present in 100 fields per region were counted. Counts are reported in Table I in number of cells per square millimeter. It has been found previously (1) that mast cells immediately under the epidermis are less numerous and show some degree of degranulation. Consequently, superficial and deep counts are now reported as separate units. Degranulated cells were also counted separately.

Determination of histamine content of specimens of skin from the face in two rats per group were made using the guinea pig ileum assay (8).*

Results

Rats injected with increasing doses of 48/80 exhibited the changes usually attributed to liberation of endogenous histamine such as erythema and edema of the extremities; these effects gradually disappeared until hardly any sign of histamine release could be seen even after the final large doses of 48/80. The effect of the treatment on the mast cell population (Table I) is apparent in Group II; counts in the face (superficial) and in the abdomen were reduced by 60% with a large percentage of cells degranulated. Mast cells in the deeper layer of the subcutaneous tissue of the face were somewhat less affected by 48/80; 40 days later they (Group IV) had regenerated and were more numerous than in the controls of the same age (Group III); in these controls the total number of subcutaneous mast cells had decreased as compared with earlier counts made after 12 days (Group I).

All animals of Group V developed the classical erythema accompanied by periods of vigorous scratching after 5 or 6 days of the magnesium-deficient diet; at the end of the experiment they showed a decrease in the number of subcutaneous mast cells of the face and the abdomen. In animals that had been treated with 48/80 (Group VI), magnesium deficiency did not induce any peripheral erythema nor scratching; the rats remained essentially normal in appearance; however, the presence of a slight white edema of the paws was noted during the last days of the experiment. The mast cell counts in these animals were the lowest of all groups; the concentration of histamine in the specimens of skin from the face was also the lowest (6.6 μg/g) as compared with the controls (32.2 μg/g) and with Group IV (29 μg/g), which had been pretreated with 48/80 and kept on the control diet. An intermediate value of 12.5 μg/g was obtained in the group that received only the magnesium-deficient diet.

Although the rats on the deficient diet did not grow as well as the controls, they showed substantial body-weight gain. The renal calcifications occurring in magnesium-deficient rats were not influenced by treatment with 48/80.

*We are indebted to Dr. A. Beaulnes of the Laboratoire de Pharmacologie, Université de Montréal, for the histamine assays.

TABLE I

Effect of a 12-day pretreatment with 48/80 on subcutaneous mast cell population in rats after 40 days of magnesium-deficient diet

Groups		No. of rats	Face*				Abdomen*		Final B. W., g
			Superficial		Deep				
			Total	Degran., %	Total	Degran., %	Total	Degran., %	
I	Controls (12 days)	3	208±3.0	22	333±5.1	1.5	88±1.3	28	123
II	48/80 (after the 12-day pretreat.)	3	83±2.5	82	223±6.8	42.0	33±0.3	69	110
III	Controls (40 days)	3	96±3.2	17	213±3.5	2.1	48±1.1	27	321
IV	48/80 (40 days after pretreat.)	4	76±2.1	15	265±3.1	3.0	36±0.2	36	346
V	Mg-def. (40 days)	4	67±3.6	21	185±2.8	4.6	37±0.2	48	248
VI	48/80 (pretreat.) + Mg-def. (40 days)	4	23±1.5	14	129±6.0	4.2	11±0.6	40	220

*Mean of counts (±S.E.) in 100 fields per region reported in cells per mm².

Discussion

The most important finding of the present experiment was the remarkably low number of mast cells observed in the magnesium-deficient rats pretreated with compound 48/80. This indicates that magnesium deficiency aggravated the well-known degranulating effect of 48/80 on mast cells. In other words, either no regeneration of mast cells occurred after the initial 48/80 treatment or magnesium deficiency further reduced this cell population. It is not possible to decide which effect is predominant because of the concomitant decrease of the mast cell population with age; such decrease has already been reported by Bates (7). In any event, as revealed by the present results and in previous work (1), there is no doubt of the importance of magnesium for the maintenance of the mast cell.

The absence of an early erythematous reaction in the magnesium-deficient rats pretreated with compound 48/80 was another interesting observation. The development of peripheral vasodilation and erythema has already been well described in the first reports on magnesium deficiency (2); however, no explanation has been offered, as yet, for this effect. According to our results the absence of erythema in the 48/80-treated rats would be related to the effect of the compound upon mast cells. Degranulation of the mast cells and decrease in the histamine content of the skin by treatment with 48/80 before induction of magnesium deficiency would remove the cause of the peripheral effects of the deficiency. The close parallelism between the histamine concentration and mast cell count of the skin would give support to this hypothesis.

It is, as yet, difficult to propose a possible mechanism whereby magnesium could influence the mast cell. Bélanger *et al.* (1) reported that after 1 week of magnesium deficiency the variations in shape and granulation of superficial subcutaneous mast cells were exaggerated. This phenomenon, which suggests increased ameboid activity towards the epidermis, appeared at a time when the peripheral vasodilation was maximal. Presumably magnesium deprivation in the rat acts like a histamine liberator inducing degranulation of mast cells with liberation of histamine and possibly serotonin. In this respect it is of interest to recall that the rabbit, which has very few mast cells (9), does not exhibit any peripheral vasodilation nor erythema on a magnesium-deficient diet (10) in contrast to the dog and rat (11), which are both normally rich in mast cells.

Acknowledgments

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References

1. L. F. Bélanger, G. A. Van Erkel, and A. Jakerow. Science, **126**, 29 (1957).
2. H. D. Kruse, E. R. Orent, and E. V. McCollum. J. Biol. Chem. **96**, 519 (1932).
3. J. F. Riley and G. B. West. J. Pathol. Bacteriol. **69**, 269 (1955).
4. J. F. Riley. The mast cells. E. & S. Livingstone, Ltd., Edinburgh. 1959.

5. D. A. ROWLEY and E. P. BENDITT. J. Exptl. Med. **103**, 399 (1956).
6. J. R. PARRATT and G. B. WEST. J. Physiol. **137**, 179 (1957).
7. E. O. BATES. Anat. Record, **61**, 231 (1935).
8. J. H. GADDUM. Pharmacology. 4th ed. Oxford University Press, London. 1953.
9. N. A. MICHELS. The mast cells. *In* Handbook of hematology. Vol. I. *Edited by* H. Downey. Hoeber. 1938.
10. H. O. KUNKEL and P. B. PEARSON. J. Nutrition, **36**, 657 (1948).
11. E. R. ORENT, H. D. KRUSE, and E. V. MCCOLLUM. Am. J. Physiol. **101**, 454 (1932).

INFLUENCE OF VARIATION IN ENVIRONMENTAL TEMPERATURE ON THE ACUTE TOXICITY OF RESERPINE AND CHLORPROMAZINE IN MICE[1]

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Abstract

The acute toxicity of chlorpromazine in mice is 18 times higher in an environment of 4° C than in a thermoneutral environment of 30° C. Under similar conditions the acute toxicity of reserpine increases about 1200 times. These findings confirm the earlier suggestion that these drugs cause hypothermia by different mechanisms of action. Increased acute toxicity of these drugs was also noticed at a higher temperature (36° C).

At 4° C, after chlorpromazine, death occurred earlier than after reserpine. Reserpine produced a transient increase in oxygen consumption (in rats) which helped maintain body temperature but resulted in a more rapid loss of heat from the body. This would explain the difference in time of death and might explain the difference in ultimate mortality between the animals treated with these two drugs.

Introduction

The object of this study was to examine the effect of the ambient temperature on the acute toxicity of chlorpromazine and of reserpine. In many species both drugs produce hypothermia at room temperature and thus have a definite effect on thermoregulation of the body (1, 2, 3). There is evidence that this action of the two drugs differs not only in degree but in mechanism. For instance, in pigeons, reserpine lowers body temperature while chlorpromazine is apparently ineffective (4). Iproniazid opposes the action of reserpine, but potentiates that of chlorpromazine. In mice, the central stimulant, lysergic acid diethylamide, has been reported to antagonize the hypothermic effects of both drugs, and to partially prevent the hypothermic action of reserpine in the pigeon (4).

Method

Groups of 10 male white mice (Connaught Research Laboratories strain) weighing between 20 and 30 g were taken from the animal colony (23 ± 2° C) and were injected intraperitoneally with varying doses of reserpine and chlorpromazine.

The reserpine solution was prepared by dissolving 10 mg of the base in 1 ml of dilute acetic acid (B.P.) and diluting the solution with distilled water to the desired volume. Chlorpromazine hydrochloride was dissolved in normal saline. The concentration of each solution or suspension was so adjusted that 0.01 ml contained the dose per gram (mouse weight) of the drug.

The mice were then exposed to temperatures of 4°, 12°, 30°, and 36° C (reserpine) and 4°, 30°, and 36° C (chlorpromazine). Control groups were injected with equivalent volumes of normal saline and exposed similarly. The

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doses of each drug were chosen in an attempt to obtain the LD_{50} dose at each temperature studied. Food and water were made available to the animals throughout the experiments. Colonic temperatures were measured in other smaller groups of mice treated similarly.

Using male rats (150 ± 20 g) bred in the laboratory (originally a Wistar strain) measurements of colonic temperature (4 cm from the anus), skin temperature (from a point near the axilla), and oxygen consumption were made at 30° C and at 4° C (5). Values obtained from animals which had received chlorpromazine (10 mg/kg) or reserpine (1 and 4 mg/kg) were compared with those from controls. Measurements were obtained during a period of 6 hours after the administration of the drugs. The laboratory stock ration (Fox Breeder Cubes, Toronto Elevators Ltd.) and water were offered ad libitum except when measurements were being made.

Results

1. The number of deaths after treatment with various doses of reserpine or chlorpromazine at the selected temperature are shown in Tables I and II. The control animals survived during the experimental period at all ambient temperatures.

TABLE I

Reserpine, 10 mice per group, intraperitoneally

Dose (mg/kg)	No. of deaths at 24 hr (ambient temperature)			
	4° C	12° C	30° C	36° C
0.0100	0			
0.0115	1			
0.0133	4			
0.0154	6			
0.0178	9			
0.0213	10			
0.56		0		
0.65		1		
0.75		3		
0.87		7		
1.00		10		
5.62				0
7.50				2
10.00			0	5
13.33			1	9
17.80			4	10
23.72			8	
31.60			10	

The cause of death was not ascertained definitely, but appeared to be from respiratory failure at high temperatures and "hypothermia" at lower temperatures. With chlorpromazine at the 4° C temperature, colonic temperatures fell steadily to hypothermic levels prior to death. With reserpine the body temperature was maintained well for several hours, but subsequently fell rapidly. With chlorpromazine deaths occurred earlier (before 6 hours usually) than with reserpine (6–15 hours).

TABLE II

Chlorpromazine hydrochloride, 10 mice per group, intraperitoneally

Dose (mg/kg)	No. of deaths at 24 hr		
	4° C	30° C	36° C
	(ambient temperature)		
7.08	2		
7.94	3		
8.91	6		
10.00	9		
25.1			1
28.1			2
31.6			6
35.5			7
39.8			10
100.0		2	
112.0		5	
126.0		3	
141.0		4	
159.0		4	
178.0		6	
200.0		7	
224.0		10	

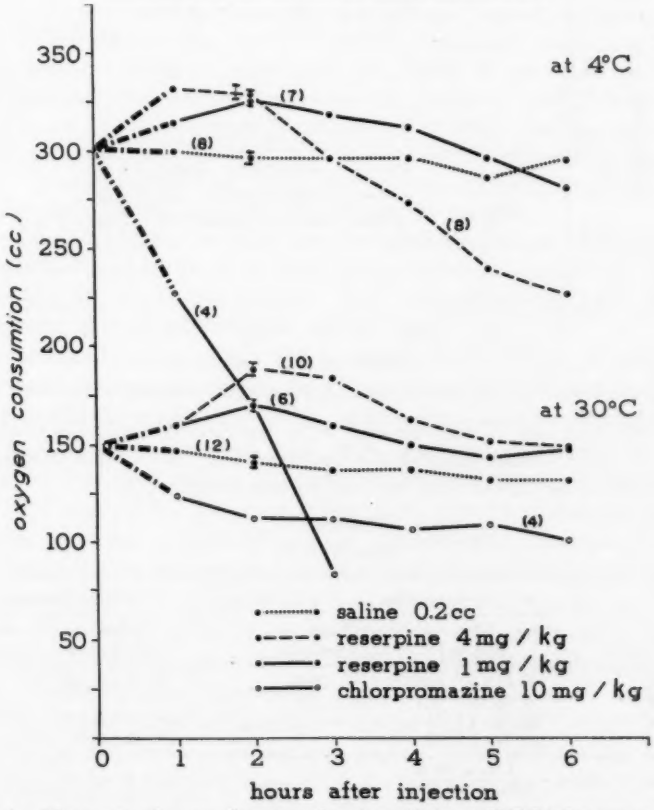

FIG. 1. Mean rate of oxygen consumption (cc/m²/min at N.T.P.) for groups of rats (number in parentheses). Standard errors indicated at 2 hours. Extrapolation from 1 hour to control value at zero.

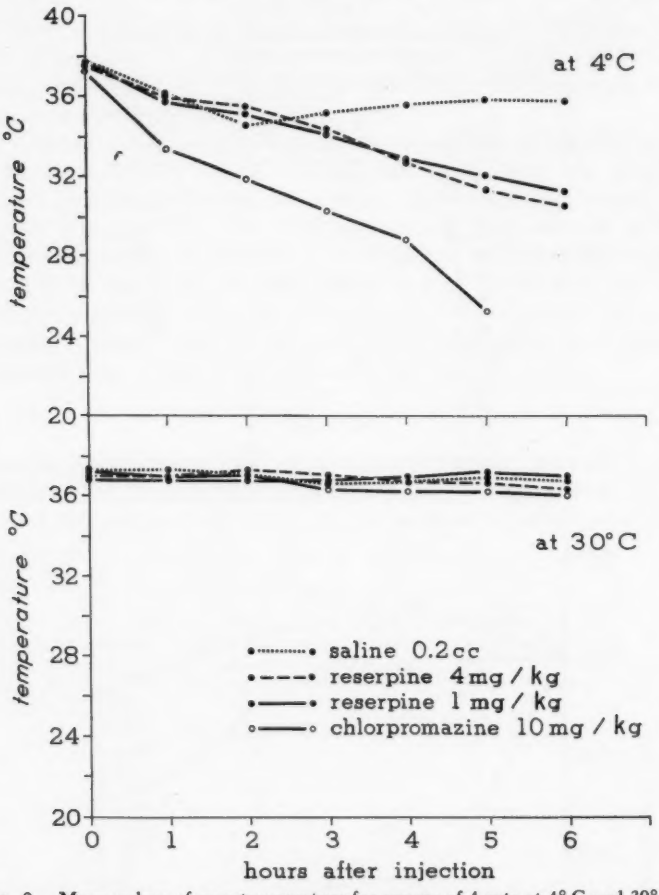

FIG. 2. Mean values of core temperature for groups of 4 rats at 4° C and 30° C.

2. At 30° and 36° C mice treated with reserpine showed no evidence of sedation, an effect observed at lower temperatures. With chlorpromazine, an initial period of excitation was followed by one of sedation.

TABLE III

Toxicity index

Ambient temp.	Reserpine		Chlorpromazine	
	LD_{50} (mg/kg)	Index of toxicity	LD_{50} (mg/kg)	Index of toxicity
4° C	0.015 ± .0007	1200	8.46 ± 0.26	18
12° C	0.80 ± .027	24		
30° C	18.84 ± 1.21	1	149.60 ± 6.44	1
36° C	10.84 ± 0.72	2	31.45 ± 0.89	5

NOTE: Index of toxicity = $\frac{LD_{50} \text{ at } 30° C}{LD_{50} \text{ at test temp.}}$.

3. The LD_{50}'s and standard error of the LD_{50}'s were calculated by Karber's method described by Finney (6). From these figures a toxicity index was determined by dividing the LD_{50} at 30° C by the LD_{50} at the test temperature (Table III).

4. The measurements of the colonic temperature and oxygen consumption of the control and treated rats are shown in graphic form (Figs. 1, 2). The skin temperatures were similar to, but a degree or two lower than, the colonic temperature at 30° C and at 4° C.

Discussion

The increase in the acute toxicity of either drug at an ambient temperature of 4° C was not unexpected. Both drugs produce hypothermia at considerably higher ambient temperatures. The way in which thermoregulation is affected by the drugs differs greatly, or one would expect somewhat similar alterations in the LD_{50}'s. In fact, the toxicity of reserpine was increased about 1200 times, while that of chlorpromazine was increased 18 times. Shemano and Nickerson (7) have observed that in curarized animals chlorpromazine induces greater hypothermia than in untreated controls or in animals treated with several other drugs, and they suggest that factors other than central suppression of thermogenesis by skeletal muscle are involved in the action. In agreement with others (3), we have found that the total metabolic rate measured at 30° C is lowered by chlorpromazine at a dose level of 10 mg/kg, and at even lower dose levels. It is also reported that vasodilatation occurs in certain areas (8) including the skin (9). These changes would all contribute to the production of hypothermia and help explain the increased toxicity of chlorpromazine.

With reserpine there is evidence that 5-hydroxytryptamine, adrenaline, and noradrenaline disappear from various tissues including those of the skin and blood vessels (10, 11, 12, 13, 14, 15). This might contribute to a cutaneous vasodilatation with consequent greater heat loss.

The experiments using rats in which oxygen consumption and colonic and skin temperatures were measured also indicate that the hypothermic actions of reserpine and of chlorpromazine differ. In agreement with the results of others (3, 4), our observations showed that, with reserpine, an increase in oxygen consumption took place shortly after administration of the drug. This recurred at both 30° C and at 4° C. However, it was found to be a transient phenomenon. At 6 hours, the oxygen consumption at 30° C is not higher than that of controls. In other experiments not reported in detail here the values at 30 hours were below those of controls. These changes in oxygen consumption are probably related to the release and depletion of adrenaline and noradrenaline produced by reserpine. Evidence bearing on this will be discussed in a subsequent paper. In spite of the transient increase in metabolic rate, the core temperature (colonic) was not elevated at an ambient temperature of 30° C and fell at 0° C. This indicates that during this period a greater heat loss from the body was taking place at both temperatures.

With chlorpromazine no increase in oxygen consumption took place at either 30° C or at 4° C. The metabolic rate (O_2 consumption) fell steadily during the 6-hour period.

The increased metabolic rate and better maintenance of body temperature for the first few hours after reserpine treatment would explain the difference in time of deaths between the animals treated with reserpine and those treated with chlorpromazine.

In a previous paper (5) it has been suggested that the precipitous fall in body temperature in small animals exposed to cold represents a failure to produce enough heat rapidly from available stores of energy. It might be suggested in the present case that a greater production of heat (as evidenced by increased oxygen consumption) in those animals treated with reserpine ultimately has a deleterious, rather than a favorable, effect on the survival of the animals. If the rate of chemical thermogenesis can only be maintained at a high level for a certain period, an increase in the total loss of heat from the body would be expected to influence the survival rate adversely.

At 36° C hyperthermia occurred in animals treated with chlorpromazine and with reserpine. This would presumably account for the increased toxicity observed in each case. The disproportionate increase might be explained by different central and local (cutaneous circulation) effects of the two drugs. The nature of these differences is not clear.

In spite of the above explanation of the difference in lethality between the two drugs, the extent of the difference seems remarkable to us. It is clear that reserpine produces definite and widespread effects at a low level of dosage. The mechanism of action is being studied further.

Acknowledgments

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References

1. A. W. Lessin and M. W. Parkes. Brit. J. Pharmacol. **12**, 245 (1957).
2. A. W. Lessin and M. W. Parkes. J. Pharm. and Pharmacol. **9**, 657 (1957).
3. R. A. Hoffman. Am. J. Physiol. **195**, 751 (1958).
4. R. A. Hoffman. Am. J. Physiol. **195**, 755 (1958).
5. E. A. Sellers, S. Reichman, N. Thomas, and S. S. You. Am. J. Physiol. **167**, 651 (1951).
6. D. J. Finney. Probit analysis. Cambridge University Press. 1947.
7. I. Shemano and M. Nickerson. Can. J. Biochem. and Physiol. **36**, 1243 (1958).
8. S. Courvoisier, J. Fournel, R. Ducrot, M. Jolsky, and P. Koetschet. Arch. intern. pharmacodynamie, **92**, 305 (1953).
9. A. B. Dobkin, G. B. Richardson, B. Gilbert, and K. I. Melville. Anesthesiology, **17**, 135 (1956).
10. P. A. Shore, S. L. Silver, and B. B. Brodie. Science, **122**, 284 (1955).
11. B. B. Brodie and P. A. Shore. Hormones, brain function and behaviour. *Edited by* H. Hoagland. Academic Press, Inc., New York. 1957.
12. M. Holzbauer and M. Vogt. J. Neurochem. **1**, 8 (1956).
13. G. P. Lewis. J. Pharm. and Pharmacol. **9**, 529 (1958).
14. J. H. Burn and M. J. Rand. Brit. Med. J. **1**, 903 (1958).
15. J. H. Burn and M. J. Rand. J. Physiol. **144**, 314 (1958).

STUDIES ON THE IN VITRO ANTITUMOR ACTIVITY OF FATTY ACIDS

II. SATURATED DICARBOXYLIC ACIDS[1]

JOSEPH F. MORGAN, SUSAN TOLNAI, AND GORDON F. TOWNSEND

Abstract

Previous studies, which showed that 10-hydroxy-2-decenoic acid from royal jelly possessed in vitro antitumor activity, have been extended to saturated dicarboxylic fatty acids. Seven of eight compounds tested in a series of chain length from C_3 to C_{10} completely prevented the development of the ascites forms of the 6C3HED lymphosarcoma, the Ehrlich carcinoma, and the TA_3 mammary carcinoma, as well as the transplantable leukemia of AKR mice. This in vitro antitumor activity could be demonstrated only at pH values below 5.0 and required admixture of the tumor cells and test compounds prior to inoculation of the mice. In general, the antitumor activity of the saturated dicarboxylic acids was found to increase progressively with increasing length of the fatty acid chain.

Previous studies from these laboratories (1, 2) have shown that admixture of royal jelly with tumor cells *before* inoculation completely suppresses the development of a transplantable mouse leukemia and the formation of ascitic tumors in mice. Fractionation studies (2) have established that most, if not all, of the antitumor activity can be attributed to the main fatty acid component of royal jelly, 10-hydroxy-2-decenoic acid. Since metabolism of this compound to some more active substance might conceivably be associated with the in vitro tumor inhibition process, and since sebacic acid has also been isolated from the same fraction of royal jelly as 10-hydroxy-2-decenoic acid (3), an investigation of the possible antitumor activity of other fatty acids has been undertaken. The present communication reports such studies with fatty acids of the saturated dicarboxylic series and demonstrates that many of these compounds exhibit in vitro antitumor activity under strictly defined conditions.

Materials and Methods

Ascitic Tumor Techniques

In vitro antitumor studies were conducted in three lines of ascitic tumors: the 6C3HED lymphosarcoma, the Ehrlich carcinoma, and the TA_3 mammary carcinoma. Stock strains of the three tumor lines which had been frozen by the procedure of Morgan, Guerin, and Morton (4) were carried by weekly serial passage in the non-specific Connaught[2] mouse line. An exception to this procedure was found desirable with the 6C3HED lymphosarcoma. Routine serial passage was carried out in C3H[2] mice but the antitumor experiments were performed in Connaught mice. Careful comparisons showed that results with the 6C3HED cells were similar in C3H and Connaught mice. The ascitic

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Contribution from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, and the Department of Apiculture, Ontario Agricultural College, Guelph.

[2]The Connaught mice used in these experiments were purchased from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario. The C3H and AKR mice were purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

material was harvested by intraperitoneal puncture 7 days after inoculation and mixed with 2 volumes of deionized water to lyse any erythrocytes; the tumor cells were removed by centrifugation, washed in Hanks' balanced salt solution (5), and resuspended in this solution to the desired cell count, as measured by a hemocytometer. This tumor cell suspension was mixed with appropriate amounts of dicarboxylic fatty acid; the pH was measured with a Beckman pH meter, and adjusted to the desired level, with 0.1 *N* HCl or 0.1 *N* NaOH. Aliquots (0.5 ml) of the reaction mixture were administered intraperitoneally to groups of at least 10 mice. Each mouse normally received five to eight million tumor cells. Groups of control mice received equivalent numbers of tumor cells subjected to the same test conditions, but without the addition of fatty acid. Male mice, 20 to 22 g in weight, were used throughout these studies.

Mouse Leukemia Techniques

In vitro antileukemia studies were carried out by an early method of Burchenal and associates (6), using female mice, 5–6 weeks of age, of the AKR strain. Leukemic cell suspensions were prepared in Gey's balanced salt solution (7) by mincing the spleens of mice dying from spontaneous leukemia. Tissue debris was removed by filtration through cotton. The leukemic cell suspensions were mixed with appropriate amounts of dicarboxylic fatty acid; the pH was measured with a Beckman pH meter and lowered to the desired level, with 0.1 *M* citric or phosphoric acid. Aliquots were then injected subcutaneously into the right side at a dose of 0.2 ml per mouse. Each mouse received two to five million tumor cells, as measured by direct count in a hemocytometer. Groups of control mice received equivalent numbers of leukemic cells subjected to the same test conditions, but without the incorporation of fatty acid.

With both types of studies, the criterion used was survival: test mice either died from the tumors or were protected. Control mice, which received the tumor cells alone, died from ascitic tumors in less than 16 days, while mice injected with the leukemic cells alone died from leukemia in approximately 20 days. One hundred per cent death of the control mice was obtained consistently with the tumors used. Mice protected by dicarboxylic fatty acids were kept under observation for 90 days from the start of the experiment. This period was found necessary to distinguish between compounds which protected the mice completely and those which afforded only partial protection by slowing the rate of tumor development. At the end of the 90-day period, the experiments were terminated but the mice were still kept under observation. No specific deaths occurred after this time. After 1 year, randomly selected mice were killed and autopsied to confirm the absence of tumors. Complete details of these experimental methods have been published (1, 2).

The dicarboxylic acids used in these studies were of the highest quality available commercially, and were employed without further purification. Highly purified 10-hydroxy-2-decenoic acid, used as a reference standard, was obtained through the courtesy of Dr. W. H. Brown, Department of Chemistry, Ontario Agricultural College, Guelph.

Results

In Vitro Antitumor Activity of Dicarboxylic Acids

Graded amounts of saturated dicarboxylic acids, in a series from malonic to sebacic (C_3 to C_{10}), were mixed with cell suspensions of the four test tumors, and inoculated into groups of mice, along with the appropriate controls. Typical results of these experiments are summarized in Table I. It is evident that all

TABLE I

Effect of dicarboxylic acids mixed with 6C3HED lymphosarcoma cells prior to inoculation of mice*

Fatty acid	Concentration (mg per ml)	Average survival (days)		Survivors (90 days)
		Test	Control	
Malonic	4.0	18	11	0/10
Succinic	1.0	17	11	0/14
	4.0	∞ †	17	9/9
Glutaric	1.0	34‡	13	7/9
	2.0	∞	14	10/10
Adipic	1.0	31‡	11	7/10
	2.0	∞	13	10/10
Pimelic	0.6	22‡	11	9/10
	1.0	∞	13	10/10
Suberic	1.0	28‡	14	2/10
	2.0	∞	9	10/10
Azelaic	0.6	31‡	11	6/10
	1.0	∞	13	10/10
Sebacic	0.4	16	15	0/10
	1.0	∞	13	10/10

*Each mouse received 5 to 8 million tumor cells either alone (control) or mixed with dicarboxylic acid (test).
†Represents survival beyond the 90-day test period.
‡Calculated from the death times of the mice which failed to survive for 90 days.

the dicarboxylic acids tested, with the exception of malonic, were able to prevent completely the development of the 6C3HED lymphosarcoma. Similar results were obtained with the Ehrlich carcinoma, the TA_3 mammary carcinoma, and the transplantable AKR leukemia. In all cases, malonic acid proved unable to prevent tumor development in concentrations equal to, or greater than, the levels effective with the other fatty acids. Experiments carried out with oxalic acid showed that this compound was partially effective but that the high concentrations required reduced the pH of the reaction mixtures to a level which, in itself, caused damage to the tumor cells. For this reason, experiments with oxalic acid were discontinued.

Relationship of pH *to Activity of Dicarboxylic Acids*

Previous studies with 10-hydroxy-2-decenoic acid (2) had shown that the in vitro antitumor activity of this compound was exerted only under acid conditions. Accordingly, effective concentrations of each dicarboxylic acid were tested at a range of pH values against the four test tumors. Typical results are presented in Table II.

It is evident (Table II) that an acid pH is required to demonstrate the in vitro antitumor activity of these compounds. The range of effective pH was found to be extremely narrow in most cases. Thus, sebacic acid was effective

TABLE II

Effect of pH on the antitumor activity of representative dicarboxylic acids mixed with tumor cells prior to inoculation of mice*

Tumor cell	Dicarboxylic acid	Concentration (mg per ml)	pH	Average survival (days)		Survivors (90 days)
				Test	Control	
AKR	Pimelic	1.6	4.5	∞ †	16	5/5
		1.6	5.5	18	12	0/4
6C3HED	Azelaic	1.0	4.3	∞	13	10/10
		1.0	5.1	13	10	0/20
	Sebacic	1.0	4.5	∞	13	10/10
		1.0	5.1	17	14	0/9
Ehrlich	Adipic	1.0	4.0	∞	13	9/9
		1.0	6.3	12	13	0/9
	Sebacic	2.0	4.5	∞	12	9/9
		2.0	6.5	13	13	0/10

*Each mouse received 5 to 8 million tumor cells or 2 to 5 million leukemia cells alone (control) or mixed with dicarboxylic acid (test).
†Represents survival beyond the 90-day test period.

against the 6C3HED lymphosarcoma at pH 4.5 but was completely ineffective at pH 5.1. Similar relationships were found with all seven dicarboxylic acids and all four test tumors. In no case was any in vitro antitumor activity demonstrable under conditions approaching neutrality.

Relationship of Concentration to Activity of Dicarboxylic Acids

Extensive experiments were carried out to determine the relationship of concentration to activity of the dicarboxylic acids, employing all seven compounds and all four tumors. Typical results are summarized in Table III.

TABLE III

Effects of varying concentrations of representative dicarboxylic acids mixed with tumor cells prior to inoculation of mice*

Tumor cell	Dicarboxylic acid	Concentration (mg per ml)	Average survival (days)		Survivors (90 days)
			Test	Control	
6C3HED	Sebacic	0.4	16	15	0/10
		0.8	20	15	0/10
		1.0	∞ †	13	10/10
Ehrlich	Glutaric	1.0	18	13	0/10
		2.0	24‡	13	6/10
		4.0	∞	11	10/10
TA_3	Suberic	1.0	20	12	0/10
		2.0	55‡	12	8/10
		3.0	∞	9	9/9

*All experiments were carried out at pH 4.5. Each mouse received 5 to 8 million tumor cells either alone (control) or mixed with dicarboxylic acid (test).
†Represents survival beyond the 90-day test period.
‡Calculated from the death times of the mice which failed to survive for 90 days.

It is apparent that a very narrow range of concentration separates the effective from the ineffective levels. Thus, with sebacic acid and the 6C3HED lymphosarcoma, a concentration of 0.8 mg per ml was completely ineffective, but a concentration of 1.0 mg per ml was completely effective. Similar relationships were found with the other fatty acids and each tumor studied.

Variation in Tumor Susceptibility

Previous experiments (2) had demonstrated that minor variations in susceptibility to 10-hydroxy-2-decenoic acid occurred among the four test tumors. Studies to test this relationship with the dicarboxylic acids are summarized in Table IV. It is evident that considerable variation does occur. Thus, the

TABLE IV

Differences in susceptibility of experimental tumors to dicarboxylic acids*

Dicarboxylic acid	Minimal effective concentrations (m*M*)			
	AKR leukemia	6C3HED lymphosarcoma	Ehrlich carcinoma	TA_3 mammary
Malonic	—	—	—	—
Succinic	—	33.8	33.8	33.8
Glutaric	—	15.1	30.2	30.2
Adipic	17.1	13.7	6.8	13.7
Pimelic	9.9	6.2	12.4	24.8
Suberic	9.7	11.4	5.7	17.1
Azelaic	13.3	5.3	10.6	10.6
Sebacic	9.8	4.9	7.4	4.9
OH-decenoic	8.0	5.3	10.6	10.6

*All experiments were carried out at pH 4.5. Each mouse received 5 to 8 million tumor cells or 2 to 5 million leukemic cells.

transplantable AKR leukemia is most sensitive to pimelic, suberic, and sebacic acids; the 6C3HED lymphosarcoma is most sensitive to pimelic, azelaic, and sebacic acids; the Ehrlich carcinoma is most sensitive to adipic, suberic, and sebacic acids; and the TA_3 mammary carcinoma is particularly sensitive only to sebacic acid. Consideration of the minimal effective concentrations for each tumor shows that, in general, the greatest activity is to be found in the compounds with the highest molecular weights, although exceptions to this order were found with all tumors studied.

Discussion

The observation that the ingredient in royal jelly active in vitro against a number of experimental mouse tumors was 10-hydroxy-2-decenoic acid (1, 2) made it of interest to determine whether such activity was a unique property of this unusual fatty acid. The present results have shown that seven of eight saturated dicarboxylic acids tested exhibited essentially similar activity under the previously established conditions of acid pH and admixture with the tumor cells prior to inoculation of mice.

A major drawback to therapeutic application of royal jelly and 10-hydroxy-2-decenoic acid has been the inability to demonstrate in vitro antitumor activity at pH values above 5.0. Similar results were obtained with the present series of dicarboxylic acids, which also failed to exhibit activity at pH levels above 5.0. It is apparent, therefore, that the activity of these compounds is dependent upon the undissociated fatty acid molecules, but no correlation could be found between the critical pH values and the dissociation constants of these compounds. It is of interest to note that previous studies on the antitumor activity

of dicarboxylic acids could not be located in the relevant literature, but early reports had recorded the activity of certain unsaturated fatty acids against some forms of transplantable mouse tumors (8, 9, 10).

A very narrow range of concentration was found to separate the completely effective from the completely ineffective levels of several of the dicarboxylic acids. This observation may possibly be related to the number of viable tumor cells in the reaction mixtures administered to the test mice. An inoculum of five to eight million tumor cells per mouse was routinely injected. It has been reported (11) that the minimum number of Ehrlich ascites cells causing 100% tumor take is in the neighborhood of 800,000, although a rate of 62% take with this tumor has been achieved with a mean inoculum of only 27 cells (12). Similar studies with the 6C3HED lymphosarcoma and the TA_3 mammary carcinoma (12) have shown a 95% incidence of tumor formation with a mean inoculum of 20 tumor cells. In the present experiments, survival of only 0.01% of the tumor cells in the reaction mixtures would still provide an inoculum of 500 to 800 viable cells, a number sufficient to initiate normal tumor formation in most cases. Accordingly, the small differences between the effective and ineffective concentrations of the test compounds in the present series may reflect the difference between 100% and 99.99% death of the tumor cells in the reaction mixtures.

As had been observed previously with 10-hydroxy-2-decenoic acid (2), considerable variation was found in the susceptibility of the four experimental tumors to the dicarboxylic acids. In general, the 6C3HED lymphosarcoma was most sensitive, followed by the Ehrlich carcinoma and the transplantable AKR leukemia, while the TA_3 mammary carcinoma was most resistant. Activity of the fatty acids against all four tumors was found to increase progressively with increasing chain length. Unexplained deviations from this generalization were noted in the sensitivity of the Ehrlich carcinoma to adipic acid, and the sensitivity of the 6C3HED lymphosarcoma to pimelic acid. On the basis of minimal effective concentrations, azelaic acid was found to be approximately equal to, and sebacic acid slightly more effective than, the reference standard of 10-hydroxy-2-decenoic acid. It would appear, therefore, that the in vitro antitumor activity of these compounds is related to the length of the fatty acid chain and that the presence of an unsaturated bond in the molecule is not essential.

In the present experiments, cell controls, without added dicarboxylic acid, were adjusted with 0.1 *N* HCl to the acid pH ranges necessary to demonstrate antitumor activity in the test mixtures. Death of the control mice within the normal 16-day period was invariably obtained at pH values from 6.9 to 3.6, but below pH 3.6 progressive damage to the tumor cells was observed. Since all subsequent studies were carried out in the range of pH 6.5 to 4.0, it is apparent that the protective effect of the test mixtures cannot be attributed to cell damage under the acid conditions of the test but must be related to an activity of the dicarboxylic acids. In this connection, it is of interest that malonic acid, which is known to be an inhibitor of the succinoxidase system in nearly all types of cells, proved completely devoid of antitumor activity.

The mechanism by which the dicarboxylic acids inhibit tumor formation has not yet been elucidated. Previous studies with 10-hydroxy-2-decenoic acid (2) have shown that the reaction between the fatty acid and the tumor cells is complete within 3 to 6 minutes, but it has not been possible to distinguish between simple adsorption on the cell surface and actual penetration into the tumor cells. Further studies with other fatty acids are now in progress to determine the relationship between chemical structure and antitumor activity and to investigate further the mode of action of these compounds.

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References

1. G. F. Townsend, J. F. Morgan, and B. Hazlett. Nature, **183**, 1270 (1959).
2. G. F. Townsend, J. F. Morgan, S. Tolnai, B. Hazlett, H. J. Morton, and R. W. Shuel. Cancer Research. In press.
3. W. H. Brown and R. J. Freure. Can. J. Chem. **37**, 2042 (1959).
4. J. F. Morgan, L. F. Guerin, and H. J. Morton. Cancer Research, **16**, 907 (1956).
5. J. H. Hanks and R. E. Wallace. Proc. Soc. Exptl. Biol. Med. **71**, 196 (1949).
6. J. H. Burchenal, R. A. Lester, J. B. Riley, and C. P. Rhoads. Cancer, **1**, 399 (1948).
7. G. O. Gey and M. K. Gey. Am. J. Cancer, **27**, 45 (1936).
8. W. Nakahara. J. Exptl. Med. **40**, 363 (1924).
9. W. Nakahara. J. Exptl. Med. **41**, 347 (1925).
10. W. Nakahara. Gann, **19**, 1 (1925).
11. G. Klein. Cancer, **3**, 1052 (1950).
12. T. S. Hauschka. Trans. N.Y. Acad. Sci. **16**, 64 (1953).

LINGCOD MUSCLE PHOSPHOMONOESTERASES

I. ACID PHOSPHATASES THAT HYDROLYZE *p*-NITROPHENYL PHOSPHATE[1]

NEIL TOMLINSON AND R. A. J. WARREN

Abstract

Five fractions (A to E), each possessing acid phosphomonoesterase activity, were separated from an aqueous extract of the muscle of lingcod (*Ophiodon elongatus*) by stepwise chromatography on diethylaminoethyl cellulose in the free-base form.

Fraction A required Zn^{++} or Mn^{++} for activity, was inhibited by heparin, and had its pH optimum at 6.0. Fraction E required Zn^{++} for activity, was not inhibited by heparin, and had its pH optimum at 5.5. Fractions B, C, and D did not require metal ions for activity, and were distinguished from each other by differences in response to pH, cysteine, ethylenediaminetetraacetate, fluoride, and tartrate.

The pH range over which fraction A was active was shifted to slightly higher values when Mn^{++} was the activator rather than Zn^{++}. Also, A was inhibited strongly by cysteine when activated by Zn^{++}, but not when activated by Mn^{++}. Data are presented that indicate these differences were due to different properties of the activating ions, rather than to the presence in fraction A of two enzymes, one activated by Zn^{++} and the other by Mn^{++}.

Introduction

Lingcod muscle contains a relatively large quantity of ribomononucleotides and other acid-soluble phosphorylated compounds (1, 2). In sterile flesh, post mortem, between 75% and 90% of the phosphorus of these compounds is released as orthophosphate. The process proceeds rapidly at room temperature and is complete in about 1 day. At 0° C, 1 to 2 weeks are required for completion (3).

Very little information is available regarding the enzymes that presumably catalyze the release of organically bound phosphorus in fish muscle post mortem (4). The occurrence of both acid and alkaline phosphatases in fish muscle has been reported (5, 6, 7), and some properties of fish muscle adenosinetriphosphatase have been described (8, 9, 10); but apart from this, no other information regarding the phosphatases of fish muscle appears to be available. An investigation of such enzymes from lingcod muscle has been undertaken, and the present paper describes the separation and some properties of five acid phosphomonoesterases from this source.

Materials and Methods

Enzyme solutions suitable for chromatography on diethylaminoethyl cellulose (DEAE)* were prepared from aqueous extracts of lingcod muscle by ammonium sulphate precipitation, followed by dialysis and lyophilization as previously described (11). The solution must be practically free from sulphate before chromatography is undertaken. In the following report an extract prepared in this fashion is referred to as a crude preparation.

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*Abbreviations: DEAE, diethylaminoethyl cellulose; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane.

Phosphomonoesterase activity was measured by determination of *p*-nitrophenol released from disodium *p*-nitrophenyl phosphate (Sigma Chemical Co.). The reaction mixture contained sodium succinate buffer of appropriate pH, 0.025 *M*, substrate 0.018 *M*, other additions as indicated, and enzyme. Controls with enzyme omitted were used. The incubation temperature was 25° C. Aliquots (0.33 ml) of the reaction mixture were removed immediately after the addition of the enzyme and again after a suitable period of incubation. These were at once mixed with 1 ml of 0.1 *N* NaOH, and the optical densities of the solutions were measured at 400 mμ. The quantity of *p*-nitrophenol formed was determined by reference to a calibration curve. When certain salts (e.g., those of manganese) were added to the reaction mixture, the precipitate which formed on addition of the alkali was removed by centrifugation before the light transmission measurement was made.

The chromatography of protein on DEAE in the free-base form, and the procedures for protein and light absorption measurements, have been previously described (12).

Results and Discussion

Preliminary observations indicated the presence of at least four acid phosphomonoesterases in the crude lingcod muscle preparations. A portion of the phosphomonoesterase activity of the crude preparation was very labile at room temperature in the absence of substrate at pH 5.5 or lower. The loss of this activity was accompanied by a marked alteration in the shape of the pH response curve. The crude preparation had a pH optimum at 5.3, whereas after the labile activity had been destroyed by adjusting the preparation to pH 4.5 and holding it for 1 hour at 25° C, the resulting preparation had a broad pH optimum between pH 3.5 and 5.0. This treatment caused the loss of over 70% of the activity of the crude preparation measured at pH 5.3, but only a very slight loss of activity at pH 3.5. These observations suggested that at least two acid phosphatases were present. Further, zinc ions were found to increase the activity of the crude preparation at values above 4.4, but to be inhibitory at pH 4.4 and lower. With zinc ions added the crude preparation and the preparation treated at pH 4.5, both exhibited the same pH optimum of 6.0. When the crude preparation was heated at 58° C for 10 minutes it appeared to lose all phosphatase activity. However, a portion of the activity could be restored by the addition of zinc ions to the preparation, and it then exhibited a pH optimum at 5.5. These additional observations appeared to be most readily explained if the presence of two more phosphomonoesterases, each activated by zinc, was assumed. In order to determine whether or not these explanations were correct, a chromatographic technique that had proved useful in the separation of other enzymes from lingcod muscle (12) was used to examine a crude preparation. The results are presented in Fig. 1.

It was found to be possible to separate the preparation into five enzymatically active fractions. These are designated A to E in the figure in accordance with the order of their elution from the column. Since a single component can be eluted in consecutive steps in the stepwise chromatographic technique (13), it

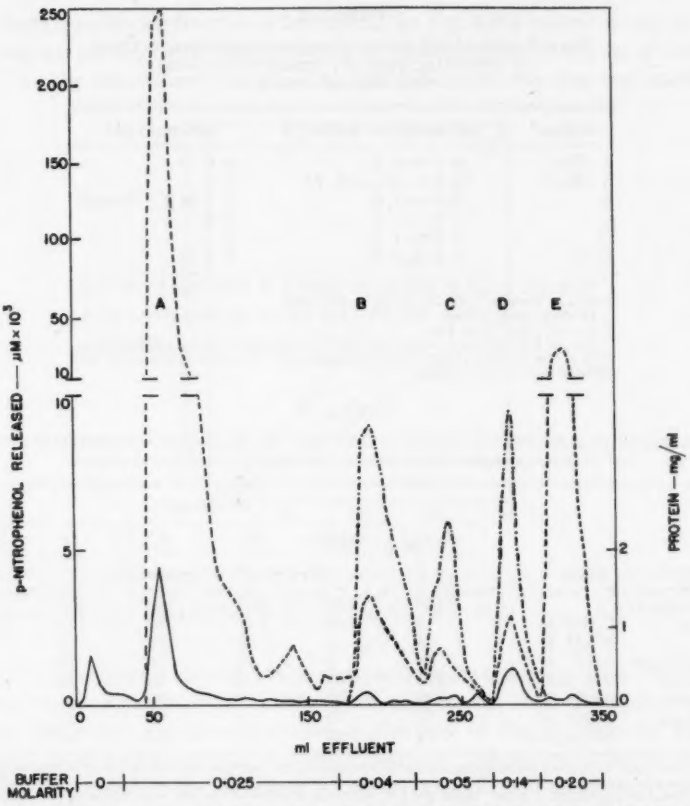

FIG. 1. Chromatographic separation of acid phosphomonoesterases from lingcod muscle. The column was 2.5 cm long by 18 mm diameter and contained 0.7 g DEAE in the free-base form. Protein applied, 63 mg. Stepwise elution, following distilled water wash, with Tris . HCl, pH 7.0 at concentrations indicated. Flow rate, 1 ml/min. Fractions, 5 ml. First fraction collected during addition of protein. Protein ———. Phosphomonoesterase activity assayed without zinc added –·–·–, with zinc added — — —. Enzyme assay system contained 0.1 ml of the fraction assayed/0.33 ml of reaction mixture, buffer pH 5.5, and $ZnSO_4$ (where indicated) 0.005 *M*. Incubation time 2 hours.

was necessary to show that the fractions had different properties before it could be concluded that they contained distinct phosphomonoesterases. In Tables I and II data are presented that support the view that each of the five fractions did indeed contain a distinct enzyme. The most marked points of difference between the fractions may be summarized as follows:

1. The influence of pH on each of the five fractions was different.
2. Fractions A and E required the addition of a metal ion for activity (see below), whereas B, C, and D did not.
3. Fractions A and E were inhibited by EDTA, whereas B, C, and D were either unaffected or stimulated by this compound.

TABLE I

The influence of pH on the phosphomonoesterase activity of chromatographically separated fractions from lingcod muscle

Fraction*	pH range for activity†	Optimum pH
A (Zn)	4.7 to 8.2	6.0
A (Mn)	5.0 to above 8.2‡	6.0
B	3.0 to 7.0	3.5 to 5.0 (broad)
C	3.0 to 7.0	5.0
D	3.0 to 7.5	5.5
E (Zn)	4.4 to 8.0	5.5

*Fractions A and E required the addition of metal ions for activity. $ZnSO_4$ or $MnSO_4$, 0.005 *M*, added as indicated.
†Buffers used: glycine. HCl, pH 2.5 to 3.6; Na succinate, pH 3.7 to 7.2; Tris . HCl, pH 7.1 to 8.2.
‡At pH 8.2 fraction A, when activated by manganous ion, exhibited about 30% of the activity shown at the optimum pH. The upper limit of pH for activity was not determined.

TABLE II

The influence of potential stimulators or inhibitors on the phosphomonoesterase activity of chromatographically separated fractions from lingcod muscle

		Fraction*					
		A(Zn)	A(Mn)	B	C	D	E(Zn)
Addition	Conc.	Activity (% of control)†					
None (control)		100	100	100	100	100	100
Cysteine	0.005 *M*	3	108	141	280	173	52
EDTA	0.022 *M*	4	0	98	279	138	1
Formaldehyde	0.17 *M*	15	5	70	36	79	8
Na fluoride	0.5 m*M*	104	103	0	46	24	102
Na fluoride	0.05 *M*	98	60	0	39	11	94
Na tartrate	0.005 *M*	52	70	1	97	21	81
Heparin	155 μg/ml	14	39	65	44	60	106

*Fractions A and E required the addition of a metal ion for activity. $ZnSO_4$ or $MnSO_4$, 0.005 *M*, added as indicated.
†Experimental conditions: Protein/0.33 ml of reaction mixture: fraction A, 50 μg; B, 6 μg; C, 3 μg; D, 22 μg; E, 4 μg. Period of incubation, 1 hour. Buffer pH 5.5. Since the activity of the enzymes, following chromatography, was rather labile in the absence of substrate at pH 5.5, they were the last component to be added to the reaction mixture.

4. Fractions A and E were more sensitive to inhibition by formaldehyde than were B, C, and D, but B, C, and D were much more sensitive to fluoride than were A and E.
5. Fraction A was activated by either zinc or manganous ions, but E was activated only by zinc (see below). Fraction A was inhibited by heparin, while E was not.
6. Fraction B was much more sensitive to inhibition by fluoride and tartrate than was C, but was only slightly affected by cysteine or EDTA, whereas C was strongly stimulated by these two compounds. Fraction D was intermediate between B and C in its sensitivity to these compounds, but tended to resemble B more closely than C.

As will be noted in Fig. 1, fractions A and E failed to show any phosphomonoesterase activity in the absence of added metal ions. The ability of various salts (each at a concentration of 0.005 *M*) to activate fractions A and E was investigated. $CaCl_2$, $CdSO_4$, $CoSO_4$, $CaSO_4$, $FeSO_4$, KCl, $NiSO_4$, $MgSO_4$, NaCl, and Na_2SO_4 did not activate either fraction. $ZnSO_4$ activated both fractions,

while $MnSO_4$ activated fraction A but not E. In Fig. 2 the course of the reaction catalyzed by fraction A in the presence of various concentrations of $ZnSO_4$ is shown. In the experiment described by this figure, the reaction was started by

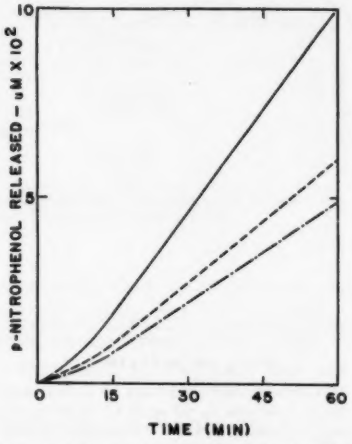

FIG. 2. The time course of the reaction catalyzed by fraction A activated by various concentrations of $ZnSO_4$. Protein, 53 μg/0.33 ml of reaction mixture. Buffer pH 5.5. Reaction started by the addition of protein. $ZnSO_4$ 0.00125 *M* —·—·, 0.005 *M* ———, 0.02 *M* — — —.

adding the enzyme to the otherwise complete mixture after a 15-minute period of temperature equilibration. Under these conditions, after the addition of the enzyme there was a gradual increase in the rate of the reaction for 10 to 15 minutes, after which the rate became constant. When the order of addition was altered so that either $ZnSO_4$ or substrate was the last component added to the mixture, the period of increasing rate was shortened to less than 5 minutes. The same course of reaction was followed at all concentrations of $ZnSO_4$ tested. Similar results were obtained with $MnSO_4$ as activator of fraction A, or with $ZnSO_4$ and fraction E. In Fig. 3 the influence of different concentrations of $ZnSO_4$ and $MnSO_4$ on the activity of the fractions is shown. The optimum concentrations of $ZnSO_4$ and $MnSO_4$ for A were about 0.007 *M* and 0.01 *M*, respectively, while for E the optimum concentration of $ZnSO_4$ was about 0.016 *M*.

The data of Table I indicate that the pH range over which fraction A showed activity was displaced a little toward the higher pH's when $MnSO_4$ was the activator rather than $ZnSO_4$, and the data of Table II show that the effects of some possible stimulators or inhibitors (14, 15, 16, 17) on the activity of this fraction varied depending on whether $ZnSO_4$ or $MnSO_4$ was the activator. At first glance these facts might be interpreted as indicating the possible presence of two different enzymes in fraction A, one activated by zinc ions, the other by manganous ions. However, the small shift in pH range for activity, and the ability of cysteine to inhibit when zinc but not when manganese was the activator, appear to be related to properties of the activating ions. Thus, when

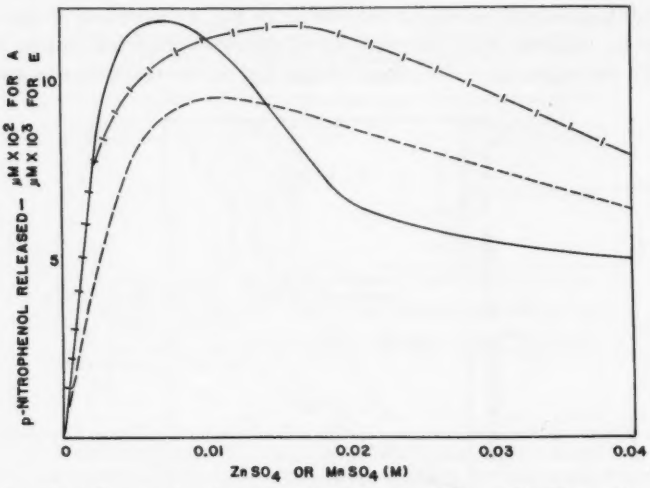

FIG. 3. The influence of various concentrations of $ZnSO_4$ and $MnSO_4$ on the activity of fractions A and E. Protein: fraction A, 65 μg/0.33 ml of reaction mixture; fraction E, 2 μg. Incubation time 2 hours. Buffer pH 5.5. Fraction A with $ZnSO_4$ ———, A with $MnSO_4$ — — —, E with $ZnSO_4$ —ı—ı.

the ability of cysteine to complex with zinc and manganous ions was investigated by means of the procedure of Martin and Edsall (18), it was found that cysteine formed a complex with zinc ion at pH 4.3 and higher, but not with manganous ion below pH 7.7. Togasawa (19) has presented spectrophotometric evidence that manganese and cysteine do not complex below pH 8.0. As the effect of cysteine on the activity of the fraction was studied at pH 5.5 the observed results could be expected even if only one enzyme was involved. Again, with regard to the small shift in the pH range for activity of the fraction, manganous hydroxide is very much more soluble than is zinc hydroxide, and this fact will account for the effect observed at the upper end of the range, while the small difference at the lower end probably reflects a difference in the ability of the two ions to complex with the enzyme. The other differences observed are quantitatively smaller, but no explanation for them can be offered — they may or may not be related to properties of the activating ions. However, direct evidence that two enzymes, one activated by zinc and the other by manganese, were not responsible for the activity of fraction A was provided (1) by the fact that when the chromatographic fractions making up fraction A in Fig. 1 were assayed with manganese as activator, rather than zinc, the results exactly paralleled those obtained with zinc as activator, thus showing that there was no tendency for the activities to separate on the column, and (2) by the data of Table III. These show that when the two ions were used together at nearly their optimum concentrations (0.005 *M* and 0.01 *M* for zinc and manganese, respectively) to activate an amount of enzyme that would limit the rate of the reaction with either of the ions separately, then their activating effects were not additive. In view of the fact that their effects were additive when

TABLE III

The influence of $MnSO_4$ and $ZnSO_4$ separately and together on the phosphomonoesterase activity of fraction A from lingcod muscle

Addition		*p*-Nitrophenol released,* $\mu M \times 10^3$
$ZnSO_4$	$MnSO_4$	
0.00125 *M*	—	14.0
—	0.0025 *M*	10.8
0.00125 *M*	0.0025 *M*	26.0
0.005 *M*	—	22.0
—	0.01 *M*	18.4
0.005 *M*	0.01 *M*	24.8

*Experimental conditions: Protein/0.33 ml of reaction mixture, 12 μg. Incubation time 30 minutes. Buffer pH 6.0.

they acted together at less than their optimum concentrations (0.00125 *M* and 0.0025 *M*), thus apparently ruling out the possibility that if two separate enzymes were involved, then the ion activating one might inhibit the other, this strongly suggests that each of the ions was not activating a separate enzyme.

The small peak of activity observed in the trailing edge of the peak of fraction A (at about 140 ml of effluent in Fig. 1) was not reproducible and did not always appear even when the same crude preparation was chromatographed on separate occasions. It is regarded as an artifact, although no explanation for its appearance can be given. The enzymatic activity of fractions A and E was completely destroyed by heating them at 100° C for 1 minute.

In Table IV the influence of various salts (each at a concentration of 0.005 *M*) on the phosphomonoesterase activity of fractions B, C, and D is recorded. The activity of the fractions was inhibited by nearly all the salts tested—even

TABLE IV

The influence of salts on the phosphomonoesterase activity of fractions B, C, and D from lingcod muscle

	Fraction		
	A	B	C
Addition*	Activity (% of control)		
None (control)	100	100	100
$CaCl_2$	98	76	91
$CdSO_4$	44	22	26
$CoSO_4$	32	5	24
$CuSO_4$	0	3	2
$FeSO_4$	50	36	61
KCl	100	67	77
$MgSO_4$	75	52	80
$MnSO_4$	97	109	112
Na_2SO_4	94	79	99
$NiSO_4$	0	8	16
$ZnSO_4$	41	50	50

*All salts added at a concentration of 0.005 *M*. Other conditions as in Table II.

KCl and Na_2SO_4 caused some inhibition—but $MnSO_4$ stimulated the activity of fractions C and D to a small extent. Although it is not shown in the table, some additional observations that appear to be of interest were made of the effects of certain combinations of inhibitors and stimulators on the activity of fractions B, C, and D. When cysteine and $MnSO_4$ (each 0.005 *M*) were added together, their separate effects on each of the fractions were found to be additive. When EDTA and sodium fluoride (the latter at 0.5 mmolar) were added together, the inhibitory effect of fluoride on fractions B and D was almost completely prevented, but fluoride inhibition of C was not prevented. When EDTA, or cysteine, and tartrate were added together, the effect of tartrate on each fraction was not altered.

In summary, the evidence clearly indicates that fraction B corresponds to the phosphomonoesterase activity in the crude preparation resistant to destruction at pH 4.5 in the absence of substrate, while C and D together correspond to the activity labile under these conditions. Fraction A corresponds to the activity of the crude preparation activated by zinc ions, but labile to heating at 58° C at pH 7.0, and fraction E to the activity activated by zinc ions and resistant to heating at 58° C at pH 7.0. There are thus a minimum of five acid phosphomonoesterases in lingcod muscle each able to hydrolyze *p*-nitrophenyl phosphate.

References

1. V. M. Creelman and N. Tomlinson. Unpublished data.
2. H. L. A. Tarr. J. Fisheries Research Board Can. **7**, 608 (1950).
3. N. Tomlinson, V. M. Creelman, and K. G. Reid. To be published.
4. H. L. A. Tarr. *In* Annual review of biochemistry. Vol. 27. *Edited by* J. M. Luck, F. W. Allen, and G. MacKinney. Annual Reviews, Inc., Palo Alto, California. 1958. p. 223.
5. T. Ono and F. Nagayama. J. Tokyo Univ. Fisheries, **45**, 153 (1959).
6. A. M. Simskaya and I. A. Karpliuk. Voprosy Pitaniya, **16**, 63 (1957).
7. C. Garcia Alfonso and E. Castellá Bertrán. Anales inst. invest. vet. (Madrid), **6**, 129 (1954).
8. S. M. V. Hunt and N. A. Matheson. Food Technol. **12**, 410 (1958).
9. W. Partmann and G. Nemitz. Z. Lebensm. Untersuch. u. Forsch. **110**, 109 (1959).
10. K. Saito and T. Hidaka. Bull. J. Soc. Sci. Fisheries, **21**, 929 (1955).
11. N. Tomlinson. Can. J. Biochem. and Physiol. **36**, 633 (1958).
12. N. Tomlinson. Can. J. Biochem. and Physiol. **37**, 493 (1959).
13. H. A. Sober and E. A. Peterson. Federation Proc. **17**, 1116 (1958).
14. M. A. M. Abul-Fadl and E. J. King. Biochem. J. **45**, 51 (1949).
15. L. M. Buruiana. Naturwissenschaften, **44**, 306 (1957).
16. B. H. J. Hofstee. Arch. Biochem. Biophys. **59**, 352 (1955).
17. E. Ito, S. Kondo, and S. Watanabe. J. Biochem. (Tokyo), **42**, 793 (1955).
18. R. B. Martin and J. T. Edsall. J. Am. Chem. Soc. **81**, 4044 (1959).
19. Y. Togasawa. Bull. J. Soc. Sci. Fisheries, **23**, 109 (1957).

THE ACETYLATION OF PROTHROMBIN[1]

RICARDO H. LANDABURU AND WALTER H. SEEGERS

Abstract

Purified bovine prothrombin was acetylated to a range of 27 to 30% of its amino groups. Except for just significant amounts it lost its power to generate thrombin-C. Upon activation the yield of thrombin-E was 100% on the basis of the thrombin-C yield that was obtained on an aliquot sample of the prothrombin before acetylation. Activation was achieved in 25% sodium citrate solution, with calcium ions plus brain extract thromboplastin, and with calcium ions plus protein-free brain thromboplastin. Evidently no Ac-globulin was needed; however, a concentrate of Ac-globulin accelerated the activation. The pH optimum for activation with brain extract thromboplastin is more to the alkaline side than with prothrombin. Activation with purified platelet factor 3 was quite slow, but was accelerated by adding a small amount of thrombin-E, in the form of acetylated thrombin, at zero time. Thrombin-C did not function in that capacity. A change in prothrombin had to be accompanied by a proportionate change in the thrombin to have the latter serve as a catalyst in the activation. Ac-globulin accelerates the hydrolysis of *p*-toluenesulphonyl-L-arginine methyl ester by thrombin. Ac-globulin owes its activity to thrombin. The view is expressed that Ac-globulin is measured as accelerated thrombin activity in the activation of prothrombin. In other words Ac-globulin accelerates the activation of acetylated prothrombin by thrombin-E. The amino groups on the N-terminal glutamic acid residues are free in the thrombin-E obtained from acetylated prothrombin. It is possible to acetylate an amino group in prothrombin that is necessary for thrombin-C activity.

Introduction

The classical thrombin derived from prothrombin has the property of clotting fibrinogen. This occurs on the basis that there is special proteolysis in which one or two peptides are split from the fibrinogen (1). Then thrombin functions as a polymerase in the polymerization of fibrin monomer (2), and with sufficient thrombin and time the fibrin gel is digested and may dissolve (2). Most generally thrombin activity is measured by the clotting of fibrinogen or by hydrolysis of *p*-toluenesulphonyl-L-arginine methyl ester (TAMe). These two assays, however, may give widely divergent results (3). In fact thrombin can lose all of its clotting power and still retain some TAMe or esterase activity. Such thrombin has been called esterase thrombin or thrombin-E. In the activation of prothrombin, thrombin-E always seems to come first (4). The recognition of thrombin-E is thus in effect the discovery of an enzyme for it is distinctly different from thrombin-C (clotting thrombin). This enzyme can readily be used extensively in our metabolism and can occur freely in our peripheral blood without having it clot; thus it may help promote keeping the blood fluid.

Thrombin-E has a close relationship to thrombin-C and a nice experimental illustration of this was found in the acetylation of thrombin. This procedure covered about 60% of amino groups including the N-terminal glutamic acids. During the acetylation the clotting power was lost, but the TAMe splitting

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power almost doubled in strength. With more extensive acetylation of non-phenolic hydroxyl groups the thrombin-E activity was also lost but this was to a certain extent a reversible process (5).

We have now had some experience with the acetylation of prothrombin and have found that the acetylation cannot be as extensive as with thrombin. The acetylated prothrombin was used to study how it can be activated. The active enzyme is thrombin-E, which is the enzyme that does not clot fibrinogen. Evidently an amino group in prothrombin is subject to acetylation and this is one that is essential for thrombin-C activity.

Materials and Methods

Most of the materials and methods used in this work have been described: (*a*) purified resin thrombin (6); (*b*) acetylated thrombin (5); (*c*) TAMe assay and fibrinogen assay for thrombin (7, 8); preparation of platelet factor 3 (9); preparation of brain extract thromboplastin (10); preparation of brain thromboplastin (11); ninhydrin reaction for amino groups (12); purification of bovine prothrombin (13), and its chromatography on Amberlite IRC-50 (XE-64) (14). N-Terminal amino acids were determined by the method of Edman (20). The Ac-globulin concentrate used in these experiments was prepared by methods we worked out during the past two years, and in due time we hope to describe this work extensively.

Acetylation of Prothrombin

The reagents used for the acetylation of amino groups in prothrombin are the same as those used in the thrombin acetylation procedure (5). The method itself is also basically the same. We give a specific example. Prothrombin, approximately 100,000 units (ca. 50 mg), was dissolved in 10 ml of distilled water and cooled to near 0° C. The same volume of sodium acetate, saturated at near 10° C, was added. The mixture was kept in an ice–salt bath and continuously stirred. Immediately after the addition of the sodium acetate, 0.1 ml of acetic anhydride was also added quickly and the time was noted. After exactly 60 seconds the protein in the mixture was precipitated with ammonium sulphate added as crystals to a concentration of 40% saturation. The precipitate was sedimented by centrifugation in the cold at 20,000 *g* and taken up in the original volume of water. The solution was dialyzed for 3 hours against several changes of water. The efficient dialysis apparatus previously described was used (13). By following these steps exactly it was possible to obtain 100% yield of thrombin-E upon activation. The prothrombin was acetylated to a range of 27 to 30% of its amino groups. This includes the amino group of the N-terminal alanine.

Potassium carbonate (1.0 *M*) was also used in place of sodium acetate. The procedure in this case was otherwise the same. The acetylation time was kept at the level of about 60 seconds.

Activation of Acetylated Prothrombin

Activation in 25% Sodium Citrate Solution

Prothrombin preparations activate in 25% sodium citrate solution (15), and the process is accelerated with thrombin (16). The molecule undergoes a progressive degradation (17) that can be inhibited at a certain stage (18, 19), and thrombin-E appears before thrombin-C. Acetylated prothrombin also undergoes self-activation in 25% sodium citrate solution (Fig. 1), but the activation sequence stops at the thrombin-E stage. On the basis of the thrombin-C yield that was obtained on a sample of the prothrombin before acetylation the yield was 100%; however, practically all of it was thrombin-E, except for 0.5% which was thrombin-C. We determined that some acetylated thrombin, added at zero time, accelerates the activation.

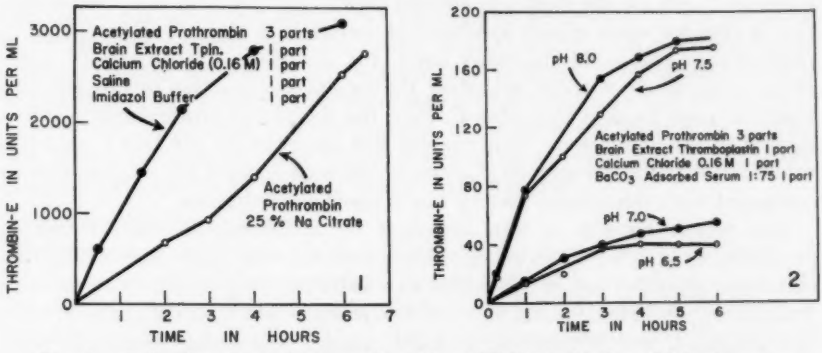

FIG. 1. Activation of acetylated prothrombin at 28° C. Activation with brain extract thromboplastin was at pH 7.25. The activity was measured with TAMe as substrate.

FIG. 2. Activation of acetylated prothrombin at 28° C. The barium carbonate adsorbed bovine serum served as a source of Ac-globulin. Activity was measured with TAMe as substrate.

Some of the activation mixture was used to get information on N-terminal amino acids. The sodium citrate was not removed and the Edman technic was used (20). Glutamic acid, which is the N-terminal amino acid in thrombin, was there in large amounts and also proline. This means that the direct acetylation of prothrombin does not alter these amino groups, a fact to be expected on the basis that the N-terminal amino acid of prothrombin is alanine (21). Direct acetylation of thrombin-C gives a product in which the amino groups of glutamic acid are blocked. This means that two kinds of acetylated thrombin have been produced. The one obtained by acetylation of thrombin-C may have up to 90% of the amino groups blocked, and this includes those on the N-terminal glutamic acid residues (5). The other, obtained by activation of prothrombin acetylated to the range of 27 to 30%, has all of its N-terminal glutamic acid residues free.

Activation with Brain Extract Thromboplastin

The ordinary brain extract thromboplastin commonly used in prothrombin time studies was added to the acetylated prothrombin. The activation con-

tinued over a period of 6 hours and the yield was about the same as in sodium citrate solution. Except for 0.9% thrombin-C the whole activity was thrombin-E (Fig. 1). The prothrombin thus loses its primary potential of becoming thrombin-C and this must be on the basis that an essential exposed amino group is acetylated. Furthermore, this is likely to be the same group that gets acetylated when thrombin-C is treated with acetic anhydride (5). The brain extract thromboplastin probably contains very little, if any, Ac-globulin and this factor is also not in the acetylated prothrombin so there is apparently no need for it.

Brain Extract Thromboplastin and pH

One of the questions we gave attention to was the optimum pH for the activation with brain extract thromboplastin. At pH 7.25 activation was rapid, but even at pH 7.0 the yield was already greatly reduced (Fig. 2). This activation is thus far more readily inhibited in a slightly acid medium than that of prothrombin (22). On the other hand, the addition of alkali can be made rather more freely without retarding the rate of the reaction or lowering the yield. It must be that thrombin-E, from the acetylated prothrombin, is functioning in the activation. This enzyme has its optimum pH at 8.8, and this would account for the shift in activation optimum toward the alkaline side as compared with thrombin-C, which has a lower pH optimum.

The details on Fig. 2 were enlarged by activating another acetylated prothrombin sample. Brain extract thromboplastin was again used and barium carbonate adsorbed serum was added as a source of Ac-globulin. The reaction was allowed to go for 2 hours at room temperature and then the concentration of thrombin-E was determined by TAMe assay. The values were as follows: 6.6 (445); 6.72 (580); 7.0 (760); 7.1 (820); 7.24 (860); 7.35 (880); 7.55 (960); 8.0 (990). The pH of the solution is given first and then the yield of thrombin in units.

In addition to working with brain extract thromboplastin we also used brain thromboplastin. This preparation is not a protein (11), but nevertheless it was heated at 100° C for 15 minutes to destroy any trace quantities of hypothetical enzymes. In the presence of this preparation and calcium ions the prothrombin all became thrombin-E within 4 hours. The brain thromboplastin looked like a homogeneous suspension when first mixed with the calcium and prothrombin. Then, as activation progressed the suspension was broken. Particles agglutinated and settled to the bottom of the tube. When this brain thromboplastin was separated by centrifugation and again suspended it looked like the original homogeneous suspension. Furthermore it was active.

Effect of Ac-globulin

Under certain conditions prothrombin is not easily converted to thrombin without an adequate amount of Ac-globulin. We doubt that there was any of this catalyst in the acetylated prothrombin and there was only a little if any in the brain extract thromboplastin. Ac-globulin is not necessary for activating acetylated prothrombin; however, an accelerator effect was demonstrated by the addition of a concentrate of Ac-globulin.

Since the acetylated prothrombin was more rapidly activated with Ac-globulin it seemed logical to us that thrombin-E of the prothrombin was having to do with the activation of prothrombin and that is was using Ac-globulin in that process. The original theory of Ac-globulin function advanced by Ware, Murphy, and Seegers (23) holds that thrombin alters plasma Ac-globulin and that the latter is a coautocatalyst. The point of emphasis in the general view advanced can be that thrombin uses Ac-globulin. If that is true then Ac-globulin is a procoagulant through the action of thrombin. From this point of view one should find that a substrate such as TAMe is hydrolyzed more rapidly by thrombin in the presence of Ac-globulin than without it, and that is what we found.

We prepared some thrombin-E in the form of acetylated thrombin and found that it had an activity of 24 units/ml on the basis of 30 minutes of TAMe hydrolysis. Then the same thrombin appeared to have 31 units/ml in the presence of 4.7 units/ml Ac-globulin concentrate, and 37 units/ml in the presence of 9 units/ml and 41 units/ml in the presence of 19 units/ml. This experiment can be interpreted on the basis that there was a direct promotion of the enzyme function by Ac-globulin, for Ac-globulin alone does not hydrolyze TAMe. This places Ac-globulin itself in the non-enzyme category of classification. The biological specificity of Ac-globulin is not enzymic.

Activation with Platelet Factor 3

To convert prothrombin to thrombin with the use of a platelet factor 3 concentrate thrombin must be added at zero time with the platelet factor 3. The reaction product is principally thrombin-C (24, 25). When thrombin is used in this way at zero time it has to be the clotting thrombin for thrombin-E does not function to promote the generation of thrombin-C under such conditions (26). The question then was whether acetylated prothrombin could be

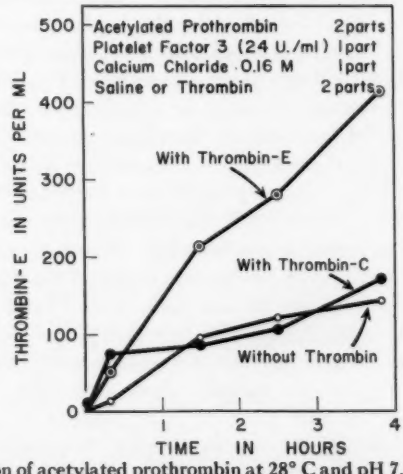

FIG. 3. Activation of acetylated prothrombin at 28° C and pH 7.25. The concentration of thrombin-E or thrombin-C was 12 units in the final mixture. Activity was measured with TAMe as substrate.

activated in this manner. The activation of acetylated prothrombin with only platelet factor 3 yielded only a little thrombin-E (Fig. 3). Thrombin-C at zero time did not accelerate this activation, but thrombin-E, in the form of acetylated thrombin, was an effective catalyst. For prothrombin it is thus thrombin-C that is needed and for acetylated prothrombin it is thrombin-E.

Discussion

The activation of acetylated prothrombin in 25% sodium citrate solution involves only acetylated prothrombin, for nothing else was present. The prothrombin was of highest purity, having been made by procedures described in this laboratory (13) and also subjected to chromatography (14) on IRC-50. We wish to attempt an interpretation of the activation in concentrated sodium citrate solution. Probably it creates conditions for breaking of labile bonds and unfolding the molecule with the result that the structure is unfolded and hence changed in physical constitution (16, 17). This involves the secondary and tertiary structure of the molecule. This occurs best when the salt concentration is so high that it is near the salting out concentration. The further activation involves the splitting of specific peptide bonds, and where is the enzyme to do this? Conceivably the thrombin-E component in the altered prothrombin is not inert just because the nonthrombin materials have not been removed as yet. The molecule undergoes self-activation, which has to be on the basis of the use of the thrombin-E activity already there. Sodium citrate solution is a medium favoring close approximation of the molecules by depressing electrostatic forces. Two peptide bonds that are easily split are the proline—x (27) and glutamic acid—x bonds (28) (where x stands for the unknown amino acid attached to the proline and glutamic acid). In this concept of self-activation the events occur in terms of the protein and its environment. After the preliminary breaking of labile bonds the catalytic arrangement of the molecule can appear and be used by neighboring molecules for further degradation.

Such a substance as brain thromboplastin is not an enzyme, but rather is seen to be a complex lipid on the basis of actual analysis (11). For the benefit of the idea that traces of an enzyme(s) might be attached we boiled the preparation for 15 minutes. This did not destroy the procoagulant. The acetylated prothrombin rapidly became thrombin-E in its presence with calcium ions. There is no more use in ascribing enzymic properties to the brain thromboplastin than to sodium citrate. The complex lipid can provide surface interfaces and points of attraction for the acetylated prothrombin. The hydrophobic bonds of the prothrombin are the likely ones to be rearranged at the lipid–water interface. That would be sufficient to unfold the molecule. Thereafter, self-activation can occur. This fully accounts for the fact that brain thromboplastin is not consumed or used up in prothrombin activation, and that there must be a certain quantity of it present, namely about one fifth the weight of the prothrombin (11).

Our finding that thrombin-E functions with Ac-globulin is of interest in connection with a previous report where we failed to get an effect (26). In the previous experiment we mixed thrombin-E with an Ac-globulin concentrate

and found that the mixture did not accelerate the activation of prothrombin. This is quite right; however, our conclusion that thrombin-E does not activate Ac-globulin is not correct. If we had used acetylated prothrombin as a substrate the activation would have been noticed. In other words we were not using an indicator when we thought we were. Ac-globulin owes its activity to thrombin and it must be the correct thrombin. The activity measured as Ac-globulin is actually *thrombin activity* accelerated by Ac-globulin in the activation of prothrombin. Thrombin-E functions with Ac-globulin in the activation of acetylated prothrombin and also in the hydrolysis of TAMe. The mechanism of Ac-globulin action is to increase the activity of thrombin. In other words Ac-globulin is a procoagulant in prothrombin activation because of the role of thrombin in the activation. In acetylated prothrombin the enzyme needed is thrombin-E (as acetylated thrombin) and with prothrombin it is thrombin-C. We thus created a proenzyme for which we also had to create an enzyme needed to function in its activation. Evidently an alteration of the proenzyme had to be accompanied by a proportionate alteration in the catalyst.

The specificity of thrombin as a catalyst in prothrombin activation is really quite evident. The change in amino groups on prothrombin upsets a possible enzyme–substrate relationship that can, however, be established if amino groups in thrombin are also altered. If they are only altered in thrombin the catalyst does not use the native prothrombin.

There must be an amino group in prothrombin that is available for acetylation and it is essential for getting thrombin-C. This group cannot be on the N-terminal alanine for this is not the N-terminal amino acid in thrombin. It could be the free amino group of lysine or arginine or both. Because of its strong basic character we think lysine is the most likely one. Furthermore this amino group must also be the one that is exposed in thrombin and becomes acetylated when the clotting activity is destroyed by acetylation.

The thrombin-E obtained from acetylated prothrombin has its glutamic acid amino groups free. When the enzyme is obtained by acetylation of purified thrombin the amino groups of glutamic acid are acetylated. The two preparations are different to that extent and presumably the amino groups under consideration have nothing special to do with the enzyme function. The special amino group is the one accessible in prothrombin.

References

1. K. Bailey, F. R. Bettelheim, L. Lorand, and W. R. Middlebrook. Nature, **167**, 233 (1951).
2. R. H. Landaburu and W. H. Seegers. Am. J. Physiol. **198**, 173 (1960).
3. R. H. Landaburu and W. H. Seegers. Proc. Soc. Exptl. Biol. Med. **94**, 708 (1957).
4. R. H. Landaburu and W. H. Seegers. Am. J. Physiol. **197**, 1178 (1959).
5. R. H. Landaburu and W. H. Seegers. Can. J. Biochem. and Physiol. **37**, 1361 (1959).
6. W. H. Seegers, W. G. Levine, and R. S. Shepard. Can. J. Biochem. and Physiol. **36**, 603 (1958).
7. S. Sherry and W. Troll. J. Biol. Chem. **208**, 95 (1954).
8. W. H. Seegers and H. P. Smith. Am. J. Physiol. **137**, 348 (1942).
9. N. Alkjaersig, T. Abe, and W. H. Seegers. Am. J. Physiol. **181**, 304 (1955).
10. A. J. Quick. The hemorrhagic disease and the physiology of hemostasis. Thomas, Springfield, Ill. 1942.
11. E. R. Hecht, M. H. Cho, and W. H. Seegers. Am. J. Physiol. **193**, 584 (1958).
12. S. Moore and W. H. Stein. J. Biol. Chem. **211**, 907 (1954).

13. W. H. SEEGERS. Record Chem. Progr. Kresge-Hooker Sci. Lib. **13**, 143 (1952).
14. K. D. MILLER. J. Biol. Chem. **231**, 987 (1958).
15. W. H. SEEGERS. Proc. Soc. Exptl. Biol. Med. **72**, 677 (1949).
16. W. H. SEEGERS, R. I. MCCLAUGHRY, and J. L. FAHEY. Blood, **5**, 421 (1950).
17. L. LORAND, N. ALKJAERSIG, and W. H. SEEGERS. Arch. Biochem. Biophys. **45**, 312 (1953).
18. N. ALKJAERSIG, E. DEUTSCH, and W. H. SEEGERS. Am. J. Physiol. **180**, 367 (1955).
19. W. H. SEEGERS and R. H. LANDABURU. Proc. Soc. Exptl. Biol. Med. **95**, 710 (1957).
20. P. EDMAN. Acta Chem. Scand. **10**, 1507 (1956).
21. W. H. SEEGERS, W. R. THOMAS, R. H. LANDABURU, and E. F. MAMMEN. Record Chem. Progr. Kresge-Hooker Sci. Lib. In press.
22. E. C. LOOMIS and W. H. SEEGERS. Arch. Biochem. **5**, 2 (1944).
23. A. G. WARE, R. C. MURPHY, and W. H. SEEGERS. Science, **106**, 2764 (1947).
24. N. ALKJAERSIG and W. H. SEEGERS. Am. J. Physiol. **182**, 347 (1955).
25. R. H. LANDABURU and W. H. SEEGERS. Am. J. Physiol. **193**, 1 (1958).
26. W. H. SEEGERS and R. H. LANDABURU. Am. J. Physiol. **191**, 1 (1957).
27. W. H. SEEGERS, R. H. LANDABURU, and W. R. THOMAS. Biochim. et Biophys. Acta, **34**, 299 (1959).
28. W. H. SEEGERS, G. CASILLAS, R. S. SHEPARD, W. R. THOMAS, and P. HALICK. Can. J. Biochem. and Physiol. **37**, 775 (1959).

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